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CHAPTER 16

Immunization Against Virus Disease

Brian R. Murphy and Robert M. Chanock

VIRAL ANTIGENS RECOGNIZED BY THE IMMUNE SYSTEM

Resistance to virus infection depends on the development of an immune response to antigens present on the surface of virions or virus-infected cells. A response to the glycoprotein or protein antigens that are present on the surface of virions or inserted into or displayed on the surface of the plasma membrane of cells is needed to generate an effective immunologically mediated elimination of virus and virus-infected cells which would otherwise initiate an unrestricted infection of the host. An immune response to the nonsurface antigens of the virus is thought to play only a minor role in resistance to the initiation of virus infection. For example, the relative importance of immunity to surface viral glycoproteins was observed in 1957 when a new subtype of influenza A virus appeared and caused a major pandemic. The 1957 Asian influenza A (H2N2) virus contained surface glycoproteins (the hemagglutinin and the neuraminidase) that were novel, i.e., the human population had not been exposed to strains containing these or related surface antigens. In contrast, the major nonsurface proteins of the new virus, the nucleoprotein and matrix protein, were closely related to corresponding antigens of previous strains of subtype H1N1 to which most individuals had been exposed. Despite this prior, and in many instances, recent experience with related nonsurface antigens, the Asian influenza A virus spread rapidly without apparent immunologically mediated restriction and caused a major pandemic. Additional evidence for the importance of the surface glycoproteins of influenza A virus in immunity is provided by passive and active immunization studies in mice. Monoclonal antibodies directed against

B. R. Murphy and R. M. Chanock: Laboratory for Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland.

the hemagglutinin (HA) or the neuraminidase (NA) protected mice against challenge with virulent wild-type virus, whereas monoclonal antibodies specific for the internal nucleoprotein or matrix protein did not alter the course of disease (12). Analysis of a set of vaccinia-recombinant viruses each expressing one of the 10 known viral gene products of the influenza A virus showed that only recombinants expressing the HA or the NA glycoprotein induced resistance to virus challenge (78). These and other observations indicate that a successful strategy of immunoprophylaxis against many virus infections requires the generation of an immune response to the surface antigens displayed on virions and on virus-infected cells. To facilitate discussion of these important proteins or glycoproteins, we will refer to them as "protective" antigens. Identification of protective antigens in the manner just described represents an important first step in the development of effective viral vaccines, whether nonliving or live.

The surface glycoproteins of lipid-containing viruses and the capsid proteins of icosahedral viruses are generally the major protective antigens, and their important role in resistance has frequently been confirmed during the characterization of monoclonal antibodies directed against these antigens. The following generalizations can be made about the nature of protective antigens and about the antibody response to them. First, and most important, antibodies predominantly recognize conformational epitopes on protective antigens. Such conformational epitopes are very difficult to mimic with peptides or other forms of an immunogen in which the protective antigenic sites are denatured. Thus, immunogens that assume the structure of native protein induce antibodies (a) that best recognize the conformational epitopes and (b) that mediate immunity most effectively. Second, antibodies recognize many antigenic sites on viral proteins. Within each antigenic site, there are multiple epitopes. For example, the parainfluenza virus attachment glycoprotein (HN) has at least six antigenic sites, three of which are recognized by neutralizing antibodies (46). After infection, not all individuals develop antibodies to each antigenic site of the infecting virus (47). After one or more reinfections, the antibody response broadens to recognize most of the antigenic sites. These findings provide a partial explanation for the failure of immunization to induce complete immunity in certain circumstances and also provide a rationale for recommending reimmunization to achieve optimal protective efficacy. Third, immunoglobulin (Ig) molecules generally do not have direct access to most of the functional sites on viral protective antigens, such as (a) the receptor-binding site; (b) sites with enzymatic activity; and (c) fusion domains, which are mostly hidden within the protein structure of the protective antigen (41). As a consequence, these sites remain relatively inaccessible to the antiviral activities of antibodies. Therefore, antibodies exert their antiviral activities by mechanisms other than direct inactivation of functional sites on the protective antigens. Instead, antibodies act directly or indirectly by other mechanisms to impede entry of virus into the cell or to prevent uncoating of virus that has entered the cell. In addition to viral structural proteins, protective immune responses can be directed at nonstructural proteins displayed on the surface of infected cells.

IMMUNE MECHANISMS THAT ELIMINATE VIRUS OR VIRUS-INFECTED CELLS

Immune mechanisms have evolved to protect the host against a diverse assortment of viral pathogens. The major purpose of the immune response is to inactivate free virus and to eliminate cells that have the potential to release infectious virus. It is important to emphasize at this juncture that the various cellular and humoral antiviral immune mechanisms function together to eliminate virus after its first encounter with the host as well as to protect the host against reinfection (179). There is considerable redundancy inherent in immunity to viruses. For example, both $CD8^+$ T cells and antibody plus complement can lyse virus-infected cells. Another well-studied example of this redundancy in immune effector mechanisms involves $CD4^+$ and $CD8^+$ T cells, which can separately effect clearance of experimental influenza A virus infection in mice. Animals lacking $CD8^+$ T cells readily clear virus from the lungs, indicating that $CD4^+$ T cells and antibody are sufficient for viral clearance (154,252). The therapeutic efficacy of antibodies acting in the absence of other immune functions has also been demonstrated in experimental influenza A virus infection of mice. Antibodies to the influenza A virus surface HA cleared virus from the lungs of persistently infected SCID mice (252). Similarly, $CD4^+$ T cell–deficient animals also readily clear influenza virus from the lungs by a $CD8^+$ T cell–dependent mechanism (154). The popular concept that $CD8^+$ T cells are solely responsible for viral clearance has been replaced with the knowledge that $CD4^+$ T cells (plus the antibodies that they provide help for) and $CD8^+$ T cells can mediate viral clearance separately and that the relative importance of these two arms of the immune response in resolution of particular viral infections varies. Even in the case of lymphocytic choriomeningitis virus (LCMV), the prototype arenavirus that had long been considered the paradigm for $CD8^+$ T cell–mediated resistance, antibodies and $CD4^+$ T cells each make a significant contribution to the resolution of primary viral infection, and antibodies play a major role in resistance to infection (15,35,146). Although it is clear that $CD4^+$ and $CD8^+$ T cells do play an important role in the resolution of viral infections, the major mediator of resistance to reinfection is antibody.

Cellular Immunity to Viruses

Natural killer (NK) cells, major histocompatibility complex (MHC) class I–restricted $CD8^+$ cytotoxic T lympho-

cytes (CTLs) and MHC class II–restricted CD4+ helper T cells (Th cells) each function independently as antiviral effector cells against viruses.

NK Cells

Humans with an inherited deficiency of NK cells experience more severe herpesvirus infections than do normal individuals (26). Persons with this deficiency eventually clear their infection in a manner comparable with that of normal persons. These observations suggest that NK cells, which do not exhibit virus-specific antiviral activity, can play an important early role in limiting the extent of certain viral infections. However, because the antiviral activities of NK cells are not virus specific, this particular arm of the cellular immune response does not represent a promising target for immunization.

CTLS

CTLS appear to be the major T-cell effectors of antiviral activity (64,99,125,193,212,285,313). The CTL receptor recognizes a short peptide derived from an endogenously produced viral protein in the context of the MHC class I β_2-microglobulin heterodimer expressed on the surface of an infected cell (164). Because the MHC class I restriction elements are present in relatively high concentration on almost all cells except neurons, CTLS can exert their antiviral effects against most infected cells. In most instances, antigen presentation by the MHC class I β_2-microglobulin heterodimer is restricted to viral peptides that are produced and processed during infection. Hence, CTLS cannot function to prevent infection, but instead are limited to the elimination of cells already infected or, alternatively, to the restriction of virus replication by the elaboration of antiviral cytokines. The net effect of CTL activity is to prevent further spread of virus and to terminate infection in cells already infected. The importance of CTLS in recovery from viral infection is indicated by the diverse strategies that viruses use to escape from CTL immunity, including (a) generation of escape mutants with missense mutations in CTL epitopes (240); (b) downregulation of the MHC class I molecules expressed on infected cells (308,311); and (c) elaboration of viral proteins that interfere with antiviral activity of cytokines, such as tumor necrosis factor, that are produced by antigen-activated CTLS (294).

The functional capabilities of CTLS have been demonstrated in many studies in which the transfer of CTL clones to infected animals resulted in significant reduction of virus replication *in vivo*. Usually, CTL antiviral activity is associated with the clearance of virus and the reduction of virus-associated pathology (158,162,193). However, disease enhancement has been observed under experimental conditions in which the quantity of CTLS transferred approximated that required to clear the virus infection (33,121).

The time course of CTL activation during viral infection in the lungs is consistent with its important role in clearance of virus infection. Primary (also called direct) CTL cytotoxic activity of pulmonary lymphocytes peaks early (day 7) during acute virus infection [such as that produced by influenza A virus or respiratory syncytial virus (RSV)] and declines to barely detectable levels by day 12 (8). Thus, restriction of virus replication mediated by CTLS is active early during the acute stage of virus infection, but this activity usually wanes rapidly by day 28 (51). Residual memory CTLS were not able to accelerate the clearance of RSV after challenge on day 45 (141). These observations suggest that the CTL component of the immune response is active primarily during the early phase of infection from the time just before peak titer of virus is reached to the time virus-infected cells are cleared, and later these cells persist in an inactive (or memory) state that does not provide resistance to reinfection (51). For viruses that replicate rapidly in the host, such as the influenza viruses and paramyxoviruses, the proliferation of memory CTLS does not appear to occur with sufficient rapidity to alter the peak titer of virus attained in the target organ (73,175,303). Because disease usually develops by the time peak titer of virus is attained, it is not surprising that immunization with antigens that predominantly induce CTL activity without an accompanying stimulation of neutralizing antibodies is less effective in restricting virus replication and preventing illness than is immunization that induces a sustained neutralizing antibody response (10,73,303).

Because of the important role of CTLS in clearance of established viral infections, the incorporation of CTL epitopes into viral vaccines has been considered an important requirement. The following characteristics of the CTL response *in vivo* make CTL epitopes less attractive as candidates for inclusion as the sole or main protective component of a viral vaccine. First, the ability of a CTL epitope to induce an immune response is MHC dependent. Because human MHC restriction elements are highly polymorphic, many protective CTL epitopes would have to be incorporated into a single vaccine to protect a large proportion of the population. Second, the duration of the primary CTL response to acute viral infections is short lived and lasts less than 2 weeks. Third, there appears to be a limited number of protective CTL epitopes in most viruses (78,141, 142). Thus, the number of protective CTL epitopes per MHC haplotype is small for many viruses. Fourth, viruses can escape from protective CTL responses by mutations within the CTL epitopes (240). Because of this, vaccinees whose resistance was induced by immunization solely with only one protective CTL epitope would be fully susceptible to infection with a CTL epitope escape mutant. Fifth, the protective effect of a CTL response can differ dramatically among CTL epitopes, some of which induce a vig-

orous protective effect against disease, whereas others induce weak protection (99). Thus, only those CTL epitopes capable of inducing a strong protective immune response could serve as suitable immunogens for inclusion in vaccines. Sixth, CTLS themselves can cause disease by an immunopathological reaction in the target organs in which virus is replicating. Thus, a fine balance exists between protective efficacy and disease-enhancing capacity (33). Seventh, the induction of CTL responses using the minimal peptide epitopes is problematic because peptides do not induce CTL responses efficiently. The induction of effective immunity requires a strong adjuvant, the cytoplasmic delivery of peptide, the expression of peptide by a viral vector, or the addition of accessory molecules such as β_2-microglobulins (99,126,236). Finally, CTLS represent a second line of defense against infection because CTLS are only effective against infected cells, whereas antibodies can neutralize the infectivity of the virus and decrease the number of cells initially infected. For these reasons, it is unlikely that CTL epitopes by themselves will replace native capsid proteins or surface glycoproteins in the formulation of viral vaccines. The induction of a CTL memory response by immunization might be helpful against viruses that replicate relatively slowly in their host or that cause persistent infection. In such situations, memory CTLS could be activated and amplified to an antiviral state and participate in the control of infection. It should be noted that live-virus vaccines induce both antibodies and CTLS efficiently, whereas inactivated or subunit virus vaccines as currently formulated are poor immunogens with respect to CTLS.

Th Cells

Th cells provide help to B cells, thereby augmenting the antibody response. However, Th cells also provide direct antiviral activity *in vivo* (172,286). The contribution to immunity made by the direct antiviral activity of Th cells appears to be less than that of CTLS. This is consistent with the more limited distribution of the restricting element of Th cells, namely the MHC class II, α, β heterodimer, which is present predominantly on lymphocytes and antigen-presenting cells such as macrophages and dendritic cells. In addition to having antiviral activity, Th cells also can mediate immunopathology (7,178,286). Viruses also seek to escape from the antiviral activities of Th cells as indicated by the finding that the Epstein-Barr virus elaborates an analog of interleukin (IL)-10 that can downregulate the production of cytokines by Th_1 cells (16).

The major mechanism by which Th cells mediate resistance to viral infection is by providing help to B cells and thereby augmenting the production of viral antibodies (252). The Th response to certain internal viral proteins may play a cooperative role in the development of effective resistance by augmenting the antibody response to a major protective antigen (144,217,243,251). For example, immunization with the membrane (M) protein of influenza A virus can prime for a subsequent augmented antibody response to the HA glycoprotein (243). The mechanism underlying this intermolecular/infrastructural cooperative effect is thought to involve the following series of events: (a) a B cell specific for the HA internalizes a virus particle containing the M and HA proteins; (b) this B cell displays processed fragments of the M protein on its class II histocompatibility proteins and is recognized by the T-cell receptor (TcR) on M-specific Th lymphocytes; and (c) signaling through contact with the TcR for an M protein epitope or the local secretion of lymphokines stimulates the HA-specific B cells displaying the M-specific antigen to divide with the resulting enhanced secretion of HA-specific antibodies. In this manner, the entire Th-cell repertoire developed against the viral surface and nonsurface proteins can be called into play to amplify a B-cell response to a protective antigen. Because Th-cell epitopes are far more numerous on antigens than are CTL-epitopes (142), the repertoire of Th cells capable of augmenting the antibody response is indeed large. For these reasons, it may be necessary to include virus structural protein antigens capable of inducing Th responses in inactivated or subunit virus vaccines may be in order to achieve maximal immunogenicity.

Humoral Immunity to Viruses

Antibodies have the major responsibility for rendering free virus noninfectious, and the principal manner in which this is achieved is by neutralization of infectivity. Antibodies can also exert antiviral activity against cells that contain viral glycoproteins inserted into the outer cell membrane. Specific human antibodies have been shown to prevent disease caused by a wide variety of viruses belonging to diverse RNA or DNA virus families that include the orthomyxoviruses, paramyxoviruses, alphaviruses, flaviviruses, arenaviruses, lentiviruses, picornaviruses, hepadnaviruses, and herpesviruses (36). Examples from clinical medicine include hepatitis A virus (HAV), measles virus, poliovirus, and varicella-zoster virus. Commercial human gamma globulin, which contains only a small proportion of antibody that is specific for any given antigen, is highly effective in prevention of hepatitis A disease and has been used widely for that purpose during the past 40 years. A large clinical trial performed in 1951 and 1952 demonstrated that human IgG was also effective in preventing paralytic disease caused by poliovirus. This important observation provided the signal that antibodies alone can confer resistance to poliomyelitis. The results of the IgG clinical trial also implied that vaccines that induced such antibodies should protect against the disease. Subsequent field trials of such immunogenic inactivated vaccines validated this view. Human IgG prepared from pooled plasma selected for a high titer of varicella-zoster virus antibodies is now licensed for use in prevention of severe varicella in immunosuppressed children.

One of the more dramatic effects of viral antibodies was observed during a study in which human IgG was shown to be effective in preventing chronic hepatitis B virus (HBV) infection in high-risk infants born to mothers who were chronically infected with HBV and who had HBV E antigen as well as HBV surface antigen (HBVsAg) in their blood (21). Almost all infants born to such mothers become chronic HBV carriers within the first few months of life. A small quantity of human IgG selected to have a high titer of HBV antibodies was 71% effective in preventing the development of persistent HBV infection when children were inoculated at birth and again at 3 and 6 months after birth (21). It appears that infection by a primate lentivirus (simian immunodeficiency virus [SIV]) can also be prevented by passively transferred antibodies derived from a healthy SIV-infected monkey that was a long-term survivor (227).

Mechanisms by Which Antibodies Decrease Viral Infectivity in vitro

Antibodies can decrease the infectivity of a virus by several distinct mechanisms. First, antibodies directed against a surface antigen can aggregate the virus particles, thereby causing a decrease in its titer. Second, antibodies acting alone or in the presence of complement can irreversibly alter the structural integrity of the virus and render it noninfectious. For example, an IgM monoclonal antibody to the HN glycoprotein of parainfluenza virus can lyse the lipid envelope of the virus particle (297), and some poliovirus monoclonal antibodies in the absence of complement can convert infectious virus particles to noninfectious empty capsids (63). Third, the major mechanism by which antibodies inactivate free virus is by neutralization in which the virus is rendered noninfectious, although its structural integrity remains largely intact. Neutralization of infectivity is achieved either by inhibiting attachment of the virus to its cell receptor (242) or preventing release of the nucleocapsid of the bound or endocytosed virus into the cell cytoplasm (67). Most neutralizing antibodies function to prevent this postadsorption step. Inhibition of attachment of virus to its cell receptor is thought to be predominantly steric in nature because most receptor-binding sites on viral attachment proteins are inaccessible to antibodies. Extensive modifications occur in the conformation of viral glycoproteins or the capsid proteins of icosahedral viruses in the events leading up to release of viral nucleic acid into the cytoplasm, and it is thought that the majority of neutralizing antibodies act to prevent successful completion of this phase in virus entry.

The very important role that neutralizing antibodies play in immunity to viral infection is highlighted by the observations that viruses use several distinct mechanisms to escape from neutralization by antibodies. Certain viruses decorate their surface proteins with *N*- or *O*-linked carbohydrates or with absorbed phospholipids to evade neutralization by antibodies (67,271). Viruses such as the influenza A virus (see chapter 41) develop missense mutations in the neutralization epitopes of the HA, i.e., the attachment protein, in an attempt to evade herd immunity of the human population conferred by previous infection with influenza A viruses of the same subtype. In addition, influenza A virus can acquire an antigenically unrelated HA from an avian influenza virus source via gene reassortment. Escape of influenza A viruses from neutralization by either mechanism allows the antigenic mutant to become the predominant circulating viral strain in the human population by displacing other less adaptive strains. Similarly, a persistent virus such as the human immunodeficiency virus (HIV) evades the humoral immune response in a single host by generating waves of antigenic viruses variants that resist neutralization by antibodies induced by their progenitors (30).

Antibodies Active Against Virus-Infected Cells

Viruses that mature at the cell surface by budding or that insert viral glycoprotein(s) into the cell surface membrane render the infected cell susceptible to lysis by humoral (antibody-dependent, complement-mediated lysis) and cellular [antibody-dependent, cell-mediated cytotoxicity (ADCC)] mechanisms. Antibodies plus complement can lyse virus-infected cells and thereby decrease the amount of infectious virus released by the cells. The importance of this phenomenon *in vivo* is indicated by the observation that complement-deficient or complement-depleted mice exhibit enhanced susceptibility to certain experimental viral infections (28,103,104). Furthermore, it was recently recognized that herpesviruses and poxviruses encode viral proteins that bind certain of the complement cascade proteins thereby decreasing the ability of the host to neutralize free virus or to lyse virus-infected cells (16,113,174). Cytophilic antibodies can arm leukocytes in the ADCC reaction, which has been shown to be important in the resistance of infant mice to herpes infection and for clearance of a retrovirus (50,136). Herpesviruses encode an IgG Fc receptor homolog, which enables the virus to evade ADCC, underscoring the *in vivo* importance of the ADCC effector mechanism (71). These mechanisms in which antibody acts cooperatively with cells or complement to exert antiviral effects *in vivo* represent, like CTLS, a second line of defense against infection because infection of cells must occur for these immune mechanisms to act effectively.

Nonneutralizing Antibodies with Antiviral Activities

Nonneutralizing antibodies can also mediate resistance to viral infections (156,255,292,314). Nonneutralizing antibodies can be directed against antigens on the surface of virus particles or against virion or nonstructural proteins displayed on the surface of infected cells. Nonneutralizing antibodies mediate antiviral activities *in vivo* by several different mechanisms. First, virus particles coated with nonneutralizing antibodies can undergo accelerated clear-

ance from the bloodstream. Second, nonneutralizing antibodies directed at surface glycoproteins such as the NA of influenza A virus or the hemagglutinin-esterase (HE) protein of coronavirus can decrease the level of virus replication in the target organ, presumably by inhibiting the release of virus from infected cells (22,314). This can be accomplished by the binding of bivalent antibodies to the newly released virus and to glycoproteins present in the plasma membrane that have not yet been incorporated into virions (22). This bivalent binding inhibits spread of virus within the target organ and results in overall reduction of virus replication. Third, nonneutralizing antibodies can lyse virus-infected cells via an ADCC mechanism (255).

Antiviral Activity of Antibodies In Vivo

Antiviral effects of antibodies *in vivo* have been studied primarily in the context of therapy rather than prophylaxis (36). However, the lessons that have been learned are relevant to prophylaxis because many vaccination strategies do not provide complete protection against infection. This means that vaccine-induced immunity must be able to contain and eradicate the low level of infection that results from breakthrough of the infecting virus. As mentioned previously, conventional wisdom had discounted the importance of antibodies in resolution of virus infections. Instead, clearance of virus was thought to be mediated primarily by CTLS. However, several recent observations cast doubt on this generalization and suggest that antibodies can participate in the resolution of virus infections and, in some instances, can independently bring about resolution of infection. The most dramatic clinical therapeutic effect of viral antibodies has been observed in patients with Argentine hemorrhagic fever, which is caused by Junin virus, an arenavirus (76). This disease carries a high mortality rate, but death can be prevented when a preparation of pooled human sera with a high titer of Junin virus–neutralizing antibodies is administered within 8 days of onset of symptoms.

In addition, antibodies can exert a therapeutic effect in privileged sites not recognized by CTLS. Central nervous system (CNS) neurons are not recognized by CTLS because these brain cells are deficient in class I MHC glycoproteins. Nonetheless, in SCID mice, persistent infection of brain neurons by Sindbis virus (an alphavirus) can be cleared rapidly by parenteral inoculation of Sindbis virus envelope glycoprotein–specific antibodies without causing obvious cell damage (151,152). The mechanism responsible for this dramatic effect is not clearly understood. However, the recent description of uptake of Igs by axons within the CNS suggests that antibodies can act within neurons to decrease replication and transcription of the viral genome and eradicate infection (79). It is also possible that antibodies might provide a signal, via viral antigens on the cell surface, that initiates an intracellular antiviral effect. Other studies in mice indicate that antibody directed against the sigma 1 outer capsid protein of reovirus (the viral attachment protein) can interrupt the spread of established reovirus infection within the CNS (292). Monoclonal antibody against the reovirus sigma 1, sigma 3, or mu 1 outer capsid protein can also inhibit the neural spread of virus from the brain to the eyes (292). A therapeutic effect of envelope glycoprotein monoclonal antibodies has also been described for neurotropic viruses that belong to two other virus families. Monoclonal antibody to the envelope glycoprotein of rabies virus (a rhabdovirus) has been reported to inhibit virus spread and viral RNA transcription in infected neural cells in culture (65). This monoclonal antibody was also active therapeutically *in vivo*. Administration of the antibody 24 hr after inoculation of virus prevented infected rats from developing lethal disease. Clearing of infection occurred even though virus had already spread to the olfactory bulb and cerebral cortex. Several observations made during this study suggested that the antibody acted intracellularly to resolve infection. Monoclonal antibodies to measles virus HA also exert a therapeutic effect in virus-infected neural cells in culture (256). Addition of these antibodies to mouse neuroblastoma or rat glioma cells persistently infected with the virus resulted in almost complete suppression of transcription and replication of the viral genome, the former occurring more rapidly than the latter.

Mucosal virus infections that are limited to the cells that line the lumen of the respiratory tract also can be cleared by specific antibodies that are delivered by parenteral inoculation or by direct instillation into the lungs. High therapeutic efficacy has been demonstrated for RSV-neutralizing antibodies delivered either parenterally or by direct instillation into the lungs of cotton rats at the height of infection (36). In the case of influenza A virus, viral HA-specific antibodies can clear the lungs of infected mice in the absence of other immune functions (252). Complete clearance of virus from persistently infected SCID mice was recently achieved using physiologic amounts of influenza A viral HA-specific antibodies inoculated parenterally. Mucosal IgA antibodies also have been shown to function within cells of another type of tissue, namely mucosal epithelium. Dimeric IgA secreted by plasma cells is transported from the basal layer of mucosal epithelial cells to their apical surface in association with the polymeric Ig receptor to which dimeric IgA initially binds (169). During its transport through Sendai virus–infected cells, virus-specific IgA can reduce intracellular virus infectivity. The mechanism responsible for this effect is not clear at this time, but it is likely that the endocytic pathway used for IgA transport and the exocytic pathway used for transport of viral glycoproteins intersect at some location within the cell.

In light of the great diversity and complexity of the *in vitro* and *in vivo* actions of antiviral antibodies, it is unlikely that simple immunogens such as peptides or antiidiotype antibodies could induce the antibody repertoire that ordinarily participates in the control of viral infections *in vivo*. This failure may limit the usefulness of such immunogens.

Antibodies Active on Mucosal Surfaces

Antibodies present in the systemic circulation protect internal organs against viruses that are introduced directly into the bloodstream (e.g., hepatitis B virus, HIV, etc.) or that spread from primary sites of replication such as the respiratory or gastrointestinal mucosa. As mentioned above, disease produced by viruses that fall into the latter category (i.e., measles, polio, hepatitis A, rubella, smallpox, and varicella) can be prevented or modified by Ig prophylaxis, often with small amounts of antibody that are difficult to detect in the blood of recipients (36). In contrast, antibodies present in the systemic circulation do not provide efficient protection against disease caused by virus infection limited to mucosal surfaces unless these antibodies are present in high titer. This is because only a small proportion of such Ig molecules move to the lumenal surface of the mucosal epithelium by transudation (36,194, 292). Therefore, to prevent viral diseases that are limited to mucosal tissue, two different strategies are required: special attention should be given to the induction of secretory antibodies produced at this site, as well as to the stimulation of a high titer of protective serum antibodies.

By passive diffusion, IgG antibodies present in the blood can gain access to mucosal surfaces, where these Ig molecules can exert antiviral activity (36,194). A gradient exists regarding the ability of serum IgG-derived antibodies to restrict virus replication on mucosal surfaces; this gradient is lung > nasopharynx > lower intestinal tract. IgG antibodies, if present in the serum in high enough titer, can provide almost complete resistance to pulmonary replication of RSV, but resistance in the upper respiratory tract is more difficult to achieve (36). Although passively transferred IgG antibodies can provide mucosal immunity in the lower respiratory tract, the major mediators of resistance to viral infection of the upper respiratory tract and the larger airways of the lungs are IgA antibodies, which are selectively transported across mucosal surfaces to exert antiviral effects on the lumenal surface. IgA antibodies are also important in the gastrointestinal tract. IgA antiviral antibodies function to clear viral infections, to modify the severity of disease on reinfection, and to prevent infection on reexposure to virus. IgA antibodies recognize the same protective antigens as IgG antibodies and the greater role of IgA versus IgG antibodies in mucosal immunity largely reflects the higher levels of IgA antibodies present on the lumenal surfaces where these antibodies are deposited by the polymeric-IgA receptor-mediated transport system. The primary mucosal IgA response peaks within the first 6 weeks of infection and usually decreases to a low, often barely detectable, level by 3 months. The transient nature of the primary mucosal antibody response probably explains the ability of many viruses to repeatedly reinfect mucosal surfaces. The IgA response to immunization is greatest at the site of immunization, suggesting that mucosal immunization is achieved most successfully by antigenic stimulation of sites directly involved in viral replication. The appearance of IgA antibodies in mucosal secretions coincides with the cessation of virus excretion during mucosal viral infections in both the respiratory and gastrointestinal tracts, and IgA antibodies have been identified as the major mediators of mucosal immunity (168,194). In order to achieve optimal resistance to virus infection in mucosal tissues, immunization must induce IgA antibodies efficiently. This is accomplished most effectively by infection of the mucosa with a live-virus vaccine.

Adverse Effects of Antibodies

Antiviral antibodies are not always helpful in the elimination of virus infection and can, in certain circumstances, augment viral infectivity and participate in the potentiation of disease. It has been proposed that heterotypic antibodies to the envelope glycoprotein of dengue virus play a central role in the severe clinical entity known as "dengue hemorrhagic fever/shock syndrome" (98). Dengue viruses replicate primarily in cells of the mononuclear phagocyte lineage. Subneutralizing amounts of dengue virus antibodies enhance dengue virus infection in these cells *in vitro* by increasing uptake of virus via IgG Fc receptors present on these cells (98,223). As a consequence, replication of virus in phagocytic cells *in vitro* is enhanced by antibody, presumably by increasing the number of cells infected as well as the amount of virus taken up by each infected cell. It has been proposed that this mechanism also plays a role in the enhanced disease seen in immunized cats challenged with infectious peritonitis virus (55). As a consequence, special attention should be given to the functional activity of antibodies induced against viruses that replicate in cells that bear receptors for the Fc fragment of IgG. Development of antibodies that bind to viral surface antigens but fail to neutralize infectivity effectively could render the vaccinee more susceptible to infection as well as to enhancement of disease once infection has occurred.

OBSTACLES TO IMMUNIZATION IN EARLY LIFE

Viruses that cause disease in early infancy present special problems for the development of effective vaccines. For example, the peak incidence of severe disease caused by RSV is at 2 months of age. For a vaccine to be effective against this virus, it must be administered to infants by 1 month of age. Because RSV is a ubiquitous pathogen and reinfection occurs commonly, most young infants possess a relatively high serum titer of passively acquired, maternally derived RSV-neutralizing antibodies. Both immunologic immaturity and a high level of maternally derived antibodies can suppress the immune response of the young infected infant to the F and G glycoprotein protective antigens of RSV (132,195). In experimental ani-

mals, passively administered RSV antibodies had adverse effects, both quantitative and qualitative, on the immune response to RSV glycoproteins expressed by a vaccinia-F (vac-F) or vac-G recombinant virus (198). For example, the development of serum antibodies to the F and G protective antigens was markedly suppressed when RSV immune serum was administered to animals the day before they were infected with vac-F and vac-G (198). Furthermore, antibodies to the RSV protective antigens that did develop had abnormally low neutralizing activity. Immunized animals that had their immune response suppressed by passively transferred antibodies were more susceptible to challenge infection with RSV than were animals that received control serum lacking RSV antibodies. Fortunately, this form of immunosuppression can be overcome, in part, by topical immunization in the respiratory tract (196).

A second important effect of maternally acquired antibodies on immunization is their ability to restrict replication of a live attenuated virus, thereby decreasing its immunogenicity (260). This is a well-known phenomenon that adversely affects the efficacy of parenterally administrated live attenuated measles virus vaccine during infancy and constitutes the basis for delaying immunization until 15 months of age, when sufficient decay of maternally acquired neutralizing antibodies has occurred. Similarly, live RSV, inoculated parenterally, readily infects and immunizes seronegative animals or humans, but it is not immunogenic in the presence of low levels of passively acquired neutralizing antibodies (25). Suppression of the replication of parenterally administered measles vaccine virus by maternally acquired measles antibodies can be overcome by administration of live vaccine by the aerosol route (247). This approach represents an alternative strategy for prevention of measles in developing countries where the disease often occurs during the first year of life and is a major cause of mortality.

The experimental live rhesus rotavirus vaccine exhibits mild reactogenicity for fully seronegative infants over 3 months of age (122). This effect can be abrogated significantly without losing immunogenicity by immunizing younger infants who possess passively acquired serum antibodies. In this instance a modulating effect of passively acquired serum antibodies contributes to further attenuation of a live-virus vaccine.

GOALS OF IMMUNIZATION AGAINST VIRAL DISEASES

The major goal in immunization against viruses is the prevention or modification of disease. Most viral vaccines prevent or modify disease without necessarily preventing infection. The ways in which this is achieved are diverse. Whereas most viral vaccines are administered before infection occurs, prevention of CNS disease in individuals inoculated with rabies virus by an animal bite can be achieved by administration of vaccine after infection has occurred. This is possible because the long incubation period of the disease permits the development of an effective immune response in time to modify infection and prevent disease.

The goal of immunization against rubella virus is prevention of fetal abnormalities caused by intrauterine infection. To achieve this goal, both males and females are immunized. Immunization of males has little direct benefit to the vaccinee other than prevention of the relatively mild illness caused by the rubella virus. The fetus of a vaccinated female is protected by immunization of the mother before pregnancy, and the fetus of an unvaccinated female is provided some protection by a decrease in the circulation of virus in the community as a consequence of herd immunity resulting from extensive immunization of males and females.

One of the predicted results of the widespread use of the oral live-poliovirus vaccine was the spread of the vaccine virus from vaccinees to unvaccinated contacts (246). This resulted in immunization of a significant proportion of the population that had not been fed vaccine. As a consequence, the incidence of poliomyelitis was reduced markedly in both vaccinated and unvaccinated individuals. Spread of vaccine virus has been viewed by many as a desirable property because it has conferred the benefits of immunization on many of the unimmunized. Of course, this has been a mixed blessing because of the rare occurrence of vaccine-associated paralytic disease among contacts of vaccinees. On balance, however, immunization of the unvaccinated appears to be a positive feature of the live-poliovirus vaccine.

Herpes simplex virus (HSV)-1 and HSV-2 cause latent infection in sensory ganglia that is periodically activated, resulting in development of new lesions and increased opportunity for transmission of virus. The goal of immunization against these viruses, therefore, includes prevention or marked restriction of (a) initial infection, (b) latency, and (c) recurrent disease caused by reactivation of latent infection. Recent studies in experimental animals with a live attenuated virus or a subunit vaccine suggest that these objectives can be achieved (177,268,269,306).

Most vaccines in use today are effective in preventing disease but are less efficient in preventing infection. In certain situations, prevention of infection is an absolute requirement for an effective vaccine. Two viruses that fit this category are HBV and HIV. Infection with HBV in the neonatal period is associated with a greatly increased risk of progression to the chronic carrier state that is strongly associated with chronic liver disease and hepatocellular carcinoma later in life. Therefore, a major goal of an HBV vaccine is to prevent transmission of infection from persistently infected mothers to their infants during the neonatal period. Considerable success has been achieved through the use of HBsAg vaccine in infancy. Immunization at or

shortly after birth is about 90% effective in preventing transmission of HBV from a persistently infected mother to her infant (153). Because infection with HIV appears to be uniformly fatal, the goal of immunization against this virus also has been to prevent infection. For most viruses, this is a difficult task, but for HIV this appears to be an even greater problem because it has proven difficult to induce antibodies that efficiently neutralize infectivity of both homotypic and heterotypic viruses, particularly viruses that are derived directly from infected individuals (49). Prevention of HIV infection by immunization will be difficult to achieve. Recent studies of inactivated SIV vaccines in monkeys indicate that the more modest goal of significant prolongation of clinical latency and amelioration of disease may be achievable (105).

Appropriate use of a highly effective vaccine can play an essential role in the eradication of a major disease caused by a virus that has only humans as its natural host and that lacks a nonhuman reservoir in nature. During the 1970s, this goal was achieved for smallpox after a global eradication program that ranks as one of the major public health achievements of this century (81). Also, we are very close to eradicating poliomyelitis from the remaining regions of the world where polioviruses continue to circulate widely as a consequence of mass oral vaccine campaigns of the type shown to be effective by Sabin (188,189). Significantly, this strategy was successful in the Western Hemisphere, which has been free of poliomyelitis for over 3 years. Wild-type polioviruses also have been successfully eliminated from circulation in many developed countries. In routine or mass immunization programs, resistance of the gastrointestinal tract to wild-type polioviruses is induced by immunization with attenuated vaccine virus strains, and this effectively interrupts transmission of virulent polioviruses in the community.

CURRENTLY LICENSED VIRUS VACCINES

The first vaccine used in humans, live vaccinia virus, was developed by Jenner almost 200 years ago for the control of smallpox. During the ensuing two centuries, vaccinia virus had a noble record of success in controlling this disease and played an essential role in the final eradication of smallpox. It is ironic that the origin of this most important vaccine virus strain has remained obscure. Attempts to determine the origin of vaccinia virus using modern molecular biologic techniques have yielded equivocal results. Vaccinia virus appears to be a distinct species, different from both variola and cowpox viruses (163).

Since the introduction of vaccinia virus, licensed vaccines have been developed for 15 other viral pathogens (Table 1). Several of these products also have played a major role in controlling the disease against which the vaccine was directed—notably poliomyelitis, yellow fever, mumps, measles, and rubella.

TABLE 1. *Vaccines licensed for use in the United States*

Vaccine	Nonliving virus	Live virus
Hepatitis A	+	o
Hepatitis B	+	o
Rabies	+	o
Influenza A	+	o
Influenza B	+	o
Poliovirus 1	+	+
Poliovirus 2	+	+
Poliovirus 3	+	+
Adenovirus type 4	o	+
Adenovirus type 7	o	+
Measles	o	+
Mumps	o	+
Rubella	o	+
Yellow fever	o	+
Vaccinia	o	+
Varicella	o	+

NONLIVING-VIRUS VACCINES

Nonliving-virus vaccines are available in the United States for the prevention of disease caused by influenza, poliomyelitis, rabies, hepatitis A, and hepatitis B viruses in humans (Table 1). Except in the last instance, these vaccines are prepared from virus that is grown in eggs (influenza types A and B) (14), a continuous monkey kidney cell line (poliovirus types 1, 2, and 3), or human diploid fibroblasts (rabies, hepatitis A). Virus is then inactivated with formalin. Inactivated poliovirus vaccine is highly effective in preventing disease (107,108), whereas influenza virus vaccine is only partially protective (181).

Semipurified influenza virus subunit vaccines have been developed in an attempt to increase purity and decrease toxicity. Such vaccines containing the HA and NA surface antigens of the virus are less toxic than are inactivated whole-virus vaccines, but there is no increase in protective efficacy (220,224). In fact, in unprimed individuals, influenza virus subunit vaccine appears to be less immunogenic than inactivated whole-virus vaccine, possibly because of a failure to stimulate Th-cell responses for internal and nonstructural viral antigens that can act to amplify B-cell responses to protective viral surface glycoproteins (19, 20,220).

Finally, one of the latest inactivated virus vaccines to be developed is a highly purified subunit preparation that contains the 22-nm HBsAg (138,275). The initial version of this vaccine, designed to protect against type B hepatitis, was unusual because surface antigen was purified from the blood of persons persistently infected with HBV. Although vaccine prepared in this manner was effective in preventing type B hepatitis, it was not widely used because it was very expensive as a consequence of the complex purification procedure and lengthy safety test required for its pro-

duction and certification. Recently, an effective second-generation HBsAg vaccine was developed using a recombinant plasmid to express the gene for HBsAg in yeast (3,170). Yeast-produced HBsAg is apparently not glycosylated, but it self-assembles into typical HBsAg particles and is immunogenic in humans. Yeast must be disrupted to release the antigen, but purification can be achieved by isopycnic and rate-zonal centrifugation combined with immunoaffinity chromatography. This new yeast-derived HBV vaccine is now licensed for use in humans. An inactivated HAV vaccine produced from a human diploid cell–grown virus is licensed in Europe and the United States (9).

Potential Sources of Antigens for Future Nonliving-Virus Vaccines

Major progress has been made recently by using monoclonal antibody and recombinant DNA technology to produce large quantities of purified protective viral antigens for use in immunoprophylaxis. Monoclonal antibody immunoaffinity chromatography or lectin chromatography facilitates purification of viral antigens from infected cells, while recombinant DNA technology makes it possible to express viral proteins in eukaryotic cells of yeast, insect, or mammalian origin. Use of these techniques has yielded candidate vaccines encompassing a large array of viral proteins that are capable of inducing protective antibodies in experimental animals. The production of viral proteins in prokaryotic cells has been less successful. Other approaches to immunization involve the production of synthetic peptides representing immunologically important domains of viral surface antigens and the use of antiidiotype antibodies as antigens to elicit an antiviral antibody response (11,129,150,295). Conventional techniques for producing formalin-inactivated whole-virus vaccines are still being actively pursued to improve current inactivated virus vaccines such as poliovirus (2) and to develop new vaccines for viruses such as Rift Valley fever virus, Japanese encephalitis virus, and tick-borne encephalitis virus (100,176,222).

Production of Viral Proteins in Eukaryotic Cells

Before this approach can be used for vaccine production several obstacles must be overcome. First, it is essential to produce protein with high efficiency to make this approach economically feasible. Mechanisms to enhance expression of viral proteins have been pursued focusing primarily on identification of strong promoters of gene expression and development of techniques for more efficient gene selection and amplification (147). Second, the viral proteins must be effectively separated from host cell protein and DNA. This latter requirement is of special concern because continuous cell lines constitute the most feasible substrate for production of viral proteins; therefore, potentially oncogenic, cellular DNA must be removed from the final vaccine. Recombinant DNA techniques to promote secretion of viral glycoproteins into the medium have been developed to facilitate effective separation of viral proteins from host cell proteins. The procedures used to produce and purify viral protein must be gentle enough to maintain the protein in its native state such that conformationally dependent neutralization epitopes are preserved so that protective antibodies can be induced. The following discussion of these approaches is not intended to be exhaustive in scope, but illustrative of current strategies in use or being developed.

Recombinant DNA techniques have been used to express viral proteins in yeast, insect, and mammalian cells. The usefulness of yeast has been described above for the production of the HBsAg vaccine. The yeast system is now being explored for the expression of the larger forms of HBsAg that include the pre-S domains not present in the licensed vaccine. For example, HBsAg containing the middle-S polypeptide (pre-S2) is glycosylated and assembles into particles with the size and density of HBsAg present in serum of infected individuals (112). Efforts also are underway to use the techniques of yeast genetics to achieve coupled expression, transport, and secretion of foreign proteins that do not require extensive purification from yeast proteins (68,116).

A major advance in viral antigen production has been the development of the baculovirus vector *Autographica california* nuclear polyhedrosis virus to express foreign genes in insect cells (157). As part of the natural life cycle of the baculovirus, newly produced virus is embedded in a proteinaceous viral inclusion made up of the polyhedrin protein that protects the embedded virus particles from inactivation by environmental factors. The polyhedrin protein is produced in large quantity (1 mg/10^6 cells) under the control of an active promoter. Foreign proteins placed under control of this promoter also are produced in large quantity by recombinant baculovirus. A number of baculovirus vectors have been constructed to optimize synthesis of foreign protein, and many foreign viral antigens have been expressed using the baculovirus/insect cell system (157).

Mammalian cells also have been used for the production of viral proteins that can be incorporated in a subunit vaccine. Mammalian cell cultures represent an optimal system for the production of viral proteins because their folding, transport, and processing closely approximate those that occur in the infected host. Mammalian cells have been used in two different systems for the production of viral proteins. First, cells in culture are infected with the virus, and the desired protective viral proteins are purified from cell lysates. Lysates rather than purified virus are used because infected cells usually contain a large excess of viral protein that is not incorporated into virions. Similarly, viral protein can be isolated from cells infected with a recombinant virus expressing the gene for the desired protein. There are many examples of this approach, but one of par-

ticular note is the vaccinia/T7 recombinant virus system in which cell cultures are infected with two vaccinia recombinant viruses; one recombinant expresses the foreign gene under control of the T7 bacteriophage promoter, and the second recombinant contains the T7 polymerase protein under control of a vaccinia promoter (87). Expression of the T7 polymerase (controlled by the vaccinia promoter) results in a high level of expression of the foreign viral gene (controlled by the T7 promoter). As a consequence, doubly infected cells produce large quantities of the protein encoded by the foreign gene. Second, mammalian cells can be used for the expression of proteins of viruses that (a) cannot be grown in cell culture [e.g., HBV (225,316)], (b) are poorly infectious, or (c) represent a significant biohazard and, hence, cannot be considered for use in an inactivated whole-virus vaccine (e.g., Lassa fever G protein). Examples of such mammalian cell expression systems include (a) mouse cells transformed by a bovine papillomavirus/HBsAg recombinant that expresses HBsAg (182); (b) Cos cells (a monkey cell line stably transformed by integrated SV40 sequences that express T antigen) infected with an SV40/HBsAg recombinant requiring T antigen for HBsAg expression (29); and (c) mammalian cells transfected with a plasmid containing a eukaryotic promoter, a foreign viral gene, and a selectable marker (305). In the last system described, selective medium is used that enriches the proportion of transfected cells expressing the marker protein, making it possible to select for cells expressing the foreign viral gene and to amplify plasmid copy number. In this manner, the level of expression of the foreign viral gene is increased. An example of this approach is the production of HSV gD glycoprotein by a continuous cell line of Chinese hamster ovary (CHO) dihydrofolate reductase (DHFR)-negative cells transformed by a plasmid containing the HSV gD gene, a eukaryotic promoter, and the gene for DHFR. Selection for HSV gD–transformed cells and amplification of plasmid copy number are achieved through the use of methotrexate medium. Transformed cells selected in this way produce a large quantity of HSV gD.

In each of the three eukaryotic systems described above, recombinant DNA technology can be used to introduce mutations into the expressed viral glycoproteins that facilitate subunit vaccine production. For example, the cell membrane anchor domain of viral glycoproteins can be deleted, with the result that the viral glycoprotein is secreted into the medium (268,269), thereby greatly facilitating purification of the protein. Chimeric proteins containing the antigenic portions of two protective antigens fused to form one immunogen have been produced, but the advantage of a chimeric protein over other forms of the immunogenic proteins has not been established (52,106).

Immunoaffinity chromatography, lectin chromatography, and physical separation techniques have been used to purify glycoproteins of HSV (gD, gC), Epstein Barr virus (gp340), RSV (F and G), parainfluenza virus type 3 (HN and F), and HIV (gp120) from cells infected with these viruses (13,77,177,203,230,299). In each case, the purified protein was shown to induce partial to complete resistance in animals. When directly compared in animals, resistance induced by immunization is greatest when the purified viral glycoprotein is in its native conformation (296). For three of these viruses (RSV, HSV-1, and HSV-2) the neutralizing antibody response of the immunized animals was less than that observed after virus infection (177,199). In the case of RSV, the purified glycoprotein vaccine induced a high level of antibodies that were able to bind to purified glycoprotein in an enzyme-linked immunosorbent assay (ELISA), but on a molar basis these antibodies had 10- to 100-fold less neutralizing activity than did antibodies induced by virus infection (199). In contrast, RSV glycoprotein expressed by a vaccinia virus RSV F or G recombinant induced antibodies with neutralizing activity comparable with antibodies produced by animals infected with the virus (52). Observations of this type underscore the need to use techniques for purification that yield glycoproteins that retain the capacity to induce antibodies with a high level of neutralizing activity or other desirable functional characteristics.

Viruslike Particles

The development of nonliving vaccines has been facilitated by the observation that protective antigens of a large number of icosahedral and lipid containing viruses can self-assemble into viruslike particles (VLPs) in the absence of viral nucleic acid. The capsids of most icosahedral viruses are composed of two or more proteins that assemble into one or two shells that surround the viral genome. In contrast to virions, VLPs consist of capsid proteins assembled into a similar shell-like structure, but viral nucleic acid is not present within the shell. These empty shells can display conformational epitopes that are not present on individual purified capsid proteins. Some of these epitopes are formed by the juxtaposition of parts of two different proteins. VLPs offer several advantages as immunogens. First, VLPs present conformational epitopes to the immune system in the same way as native infectious particles so that neutralizing antibodies and other protective immune responses are induced effectively. Second, many of the viruses for which immunogenic VLPs have been developed replicate poorly or not at all in tissue culture (e.g., the B19 parvovirus, the Norwalk calicivirus, or the papillomaviruses), thus precluding purified virus as immunogen. Third, because VLPs are noninfectious, inactivation with formalin is not required. For this reason, VLPs might prove to be better immunogens than are formalin-inactivated whole-virus vaccines because the deleterious effects of formalin on immunogenicity can be avoided. VLPs have been engineered successfully for viruses belonging to a wide range of virus families (17,69,93,119,133,137,186,232,239,270).

Initially, it was observed that picornavirus capsid proteins could self-assemble during the *in vitro* translation of viral RNA to yield 75S to 85S particles (219). Later studies indicated that VLPs could form *in vitro* following translation of picornavirus vRNA lacking most of the 5' noncoding region (44). Later, it was observed that expression of the open reading frames of capsid proteins of icosahedral viruses by a baculovirus or vaccinia vector in appropriate eukaryotic cells resulted in generation of VLPs (45,133,317). For picornaviruses, expression of the entire open reading frame is required to permit the proteolytic processing of the structural proteins by nonstructural viral-encoded proteases that is required for particle assembly.

VLPs often contain two separate proteins, and the relative level of incorporation of the proteins into the particles can affect immunogenicity. For example, native B19 parvovirus virions contain about 5% VP1 and 95% VP2 (17). VP2 alone expressed by recombinant baculovirus can assemble in insect cells into VLPs, but these particles do not induce neutralizing antibodies effectively. Surprisingly, VLPs consisting of 4% VP1 and 96% VP2 made during coinfection of insect cells with separate baculoviruses expressing VP1 or VP2 also fail to stimulate neutralizing antibodies effectively. However, VLPs containing 25% to 41% VP1 readily induce a high level of neutralizing antibodies. Thus, there are unforeseen and poorly understood structural and conformational constraints that affect the immunogenicity of VLPs, and these interactions must be addressed as each new VLP vaccine candidate is developed.

Protective efficacy has been demonstrated for VLPs derived from icosahedral as well as lipid-containing viruses (22,137,232,241). HBsAg VLPs were recently expressed in transgenic plants, suggesting that it may be possible to use fruits or vegetables to develop VLPs for use in immunization against human viruses (167).

Synthetic Peptides as Immunogens

During the early 1970s, the use of synthetic peptides as immunogens was studied using a synthetic icosapeptide representing the immunodominant domain of the outer coat protein of bacteriophage MS-2 (145). This peptide, conjugated to a carrier and emulsified in adjuvant, induced rabbits to produce antibodies that neutralized the infectivity of the bacteriophage when goat antirabbit Ig antibodies were added to the mixture. Although synthetic peptides were subsequently shown to induce neutralizing antibodies that did not depend on the aggregating effect of anti-Ig antibodies and to stimulate immunity to certain animal viruses *in vivo* (27), the immunity induced was less than that achieved when complete protein, inactivated whole virus or live virus was used as immunogen. The decreased immunogenicity of synthetic peptides is a consequence of their failure to mimic the form of epitopes on major neutralization sites of protective antigens, i.e., conformational epitopes formed by juxtaposition of discontinuous portions of the protein chain. Strategies to augment the immunogenicity of synthetic peptides, such as (a) inclusion of both B- and Th-cell epitopes in the peptide, (b) presentation of peptide in a noninfectious viral particle, or (c) expression of peptide in infectious virus—have not overcome the inherent deficiencies of this approach. Until this is achieved, it appears unlikely that a vaccine based solely on synthetic peptides will be forthcoming.

The recent demonstration that epitopes seen by CTLS consist of short peptides about eight to 10 amino acids in length presented in the context of the MHC class I heterodimer suggested that it might be possible to produce peptide vaccines to induce CTLS–mediated immunity. Success with such CTL–mediated vaccines has been achieved in several experimental systems. For example, a peptide in adjuvant induced CTLS and protected mice against Sendai virus infection (126). In addition, immunization with a vaccinia recombinant virus expressing a CTL epitope either in the context of the entire viral protein (99,141) or as a minigene (CTL epitope only) induced transient resistance *in vivo* (307). Immunization with vaccinia virus expressing a series of LCMV CTL epitopes (i.e., a string of beads) induced resistance to LCMV in mice of different MHC haplotypes (307). However, immunization with CTL epitopes is fraught with obstacles (see section on CTLS in this chapter). It has yet to be demonstrated that a peptide–CTL–epitope vaccine induces immunity that is more effective and more durable than that induced by a noninfectious VLP or by virus infection.

Production of Viral Proteins in Prokaryotic Cells

RNA or DNA viral genes cloned in prokaryotic cells using plasmid or bacteriophage vectors can express viral protein in these cells. For this purpose, cloned viral DNA is inserted in a plasmid downstream from a strong constitutive or inducible bacterial promoter and a ribosome binding site, thus assuring efficient expression. Insertion of the cloned viral DNA sequences downstream from the amino terminus of a bacterial gene controlled by the promoter enhances expression and facilitates purification of viral antigen in the form of an insoluble, fused bacterial-viral protein. Using this approach, FMDV VP1 was expressed efficiently in *Escherichia coli,* accounting for 17% of protein produced in transformed bacteria (134). When mixed with Freund's incomplete adjuvant, the fusion protein induced resistance in cattle and pigs to challenge with wild-type FMDV. Induction of resistance *in vivo* by bacterially expressed viral protective antigen also has been reported for herpesviruses and papillomaviruses (155,296), but in side-by-side comparisons resistance is usually best induced by protein that approximates its native conformation in the virion (296). Although an unglycosylated influenza A virus HA or rabies virus surface protein that was expressed in

E. coli induced an antibody response in mice, the resulting antibodies did not neutralize virus infectivity (61,165, 206). In general, success with this approach, as well as with the synthetic peptide approach, has been for viral proteins that have linear B-cell epitopes such as certain picornavirus capsid proteins and the HSV gD protein (304). Limited success with other viruses indicates that conformationally dependent epitopes are difficult to reproduce in the form of synthetic peptides or proteins expressed in prokaryotic cells. It should be noted that the Freund's incomplete adjuvant used to demonstrate the immunogenicity of *E. coli*–produced viral proteins in animals is not suitable for use in humans. This means that a safe, effective adjuvant acceptable for use in humans must be developed before synthetic peptides or viral proteins produced in prokaryotic cells can be used for immunization. The discussion of adjuvants is outside the scope of this chapter, but several new formulations and approaches are being considered for use in humans (95).

Antiidiotypic Antibodies as Immunogens

Another approach to induction of viral neutralizing antibodies involves the use of antiidiotypic antibodies as immunogens. Infection or immunization with a virus induces a neutralizing antibody (so-called antibody 1), which bears on its Fab variable region combining site a reverse image of the virion protein that is unique to that particular antibody. This unique region of the Fab is called an idiotype. Immunization with antiviral antibody (antibody 1) induces antiidiotype antibody (antibody 2), which has a conformation at its combining site that mimics the structure of the original viral antigen. These antiidiotype antibodies can then be used instead of the viral antigen to elicit an immune response (antibody 3) to the viral protein. Immunization with antiidiotype antibody has induced a neutralizing antibody response for reovirus, poliovirus, rabies virus, Coxsackievirus, or HBsAg (92,171,231,295), and protection of experimental animals has been demonstrated for poliovirus (295) and HBV (129). However, the level of neutralizing antibodies induced by these antiidiotype antibodies has been less than that elicited by infection or immunization with inactivated viral particles. Thus, an advantage of this approach to vaccine development over conventional approaches remains to be demonstrated.

Immunization with DNA Encoding Viral Protective Antigens

A new and potentially useful strategy for immunization involves direct inoculation of plasmid DNA encoding viral protective antigens into tissues *in vivo* (277,309). Expression of the protective antigen is driven by a strong promoter capable of efficient activity in a variety of mammalian cell types. The feasibility of immunization with DNA was demonstrated by the induction of antibodies to human growth hormone in mice immunized with a plasmid encoding this foreign protein. Subsequently, immunization with DNA encoding viral antigens from influenza A virus (235,293), HIV (300), human T-cell leukemia virus type 1 (5) or herpesvirus (58) has induced functional (i.e., neutralizing or syncytium-inhibiting) antibodies and/or resistance to viral infection (57,70,235,293).

Immunization with DNA has several advantages over immunization with purified viral antigens. The most important advantage is that viral antigens such as viral glycoproteins are expressed on the surface of the transfected cells and are presented to the immune system in a native state. During the process of viral antigen purification, assembly of VLPs, or inactivation of viruses with chemicals, the integrity of epitopes on protective proteins can be adversely modified, resulting in altered and weakened immunogenicity. DNA immunization, which circumvents this problem, more closely resembles immunization with a live-virus vaccine than with a nonliving antigen. Thus, viral antigens encoded by DNA are presented efficiently in the context of class I MHC antigens and are able to induce CTLS (102,293), thereby stimulating a balanced immune response more similar to infection-induced immunity than immunity induced by administration of preformed viral antigens. A second advantage of immunization with DNA is its ability to transfect cells without restriction by passively acquired viral antibodies. Whereas live attenuated vaccine virus, such as that present in the measles virus vaccine, is effectively rendered noninfectious by passive antibodies, DNA immunization should not be affected similarly. Finally, it has been observed that the foreign antigen can be expressed *in vivo* for several months after DNA immunization (312). If transfected cells can present antigens over a longer period of time than live- or nonliving-virus vaccines, more durable B- and T-cell responses might result. To date, immunization with complementary DNA (cDNA) has induced lower titers of serum antibodies than virus infection (57,90), but the technology is evolving rapidly and this form of immunization has not been optimized.

Two concerns specific to DNA immunization should be addressed as this new technology is being developed. First, viral antigens will be expressed in cells that do not normally express these antigens (e.g., muscle cells expressing influenza antigens), and it is possible that such cells will be destroyed by a humoral or CTL immune response and that an antiself immune response could be generated in the process. Second, the cDNAs might be inserted into host DNA with possible adverse consequences. A number of factors should be considered in evaluating the possibility of such insertional mutagenesis. The plasmid DNAs lack specific mechanisms for integration (in contrast to retrovirally mediated gene transfer), and this would serve to decrease the frequency of integration into host DNA. It is likely that the number of host cells that are actually transfected is small. However, if a cDNA vaccine is widely used,

the number of people immunized will be large, thereby increasing the possibility that an adverse event could occur in the immunized group. The risk of adverse consequences of insertional mutagenesis after immunization with DNAs is probably small, but this possibility should be evaluated extensively as this new technology is being developed.

Advantages of Nonliving-Virus Vaccines

Inactivated virus vaccines offer the advantage of immunization with little or no risk of infection. Rarely do such vaccines contain a contaminating adventitious residual agent or infectious vaccine virus that has resisted inactivation. On occasion, failure to inactivate vaccine virus has had serious consequences. For example, paralytic disease was produced by some of the early lots of inactivated poliovirus vaccine that contained residual infectious virus (204,221). This problem was solved very quickly by the development of more rigorous methods for detecting residual infectivity and by modifications in vaccine manufacture that ensured production of monodispersed suspensions of poliovirus. Contamination by an infectious adventitious agent was also detected retrospectively in some early lots of inactivated poliovirus. This was of some concern because the contaminating simian virus, SV40, was oncogenic in hamsters (109). Fortunately, long-term follow-up of individuals who received SV40-contaminated vaccine parenterally during early infancy failed to show evidence of an oncogenic effect (191). Live vaccinia virus was present in a preparation of inactivated vaccinia–HIV recombinant virus used to augment immunity in HIV-infected subjects. Unfortunately, an immunodeficient vaccinee developed progressive vaccinia initiated by residual vaccinia virus present in the incompletely inactivated vaccinia-HIV recombinant virus (315). The use of purified viral proteins or synthetic peptides would eliminate the possibility of both types of contamination. In addition, the preparation of vaccines free of nucleic acid precludes the possibility of long-term adverse effects associated with infection or integration of viral nucleic acid into the host genome.

Disadvantages of Nonliving-Virus Vaccines

Several nonliving-virus vaccines have potentiated disease rather than prevented it (88,123,130). This was first observed with formalin-inactivated measles virus vaccine (88). Initially, this vaccine prevented measles, but after several years vaccinees lost their resistance to infection. When subsequently infected with naturally circulating measles virus, the vaccinees developed an atypical illness with accentuated systemic symptoms and pneumonia (88,202,229). Retrospective analysis showed that formalin inactivation destroyed the ability of the measles fusion (F) protein to induce hemolysis-inhibiting antibodies, but it did not destroy the ability of the H (hemagglutinin or attachment) protein to induce neutralizing antibodies (209,210). Thus, vaccinees developed an unbalanced response that included H protein immunity but not F protein immunity. When the immunity induced by the H protein had waned sufficiently to permit extensive infection with measles virus, an altered and sometimes more severe disease was seen at the sites of measles virus replication (23,31). The immune mediators of this atypical disease have not been defined completely. A differential effect of formalin on mumps and parainfluenza virus surface glycoproteins also has been observed; F protein immunogenicity is greatly decreased, whereas some immunogenicity of HN protein is retained (211,218). Chemical inactivation of poliovirus for use in a vaccine results in modification of a major neutralization epitope, indicating that inactivation of the infectivity of icosahedral as well as lipid-containing viruses can modify the antigenicity of a vaccine so that vaccinees might develop an altered immune response (82).

Potentiation of disease also was observed after parenteral administration of formalin-inactivated RSV (FI-RSV) vaccine (123,130). In clinical trials conducted in the mid-1960s, a FI-RSV vaccine induced a measurable serum-neutralizing antibody response but did not protect the young vaccinees. Unexpectedly, upon subsequent natural infection with RSV the FI-RSV vaccinees developed severe RSV lower respiratory tract disease significantly more often than did infants and young children who had received an inactivated parainfluenza type 1 virus vaccine or an inactivated trivalent parainfluenza virus vaccine (123,130). Speculation on the mechanism of disease potentiation by the inactivated RSV vaccine has centered on several possible aberrations of the immune response to vaccine that involve an imbalance between or within various compartments of the immune system (38). First, it is likely that the vaccine failed to induce appreciable local respiratory tract RSV secretory IgA antibodies, thereby leaving vaccinees susceptible to infection under conditions in which the adverse effects of a series of other unbalanced responses might exacerbate disease. Second, in addition to an imbalance between systemic and local secretory antibodies, it is clear that there was an imbalance within the systemic Ig response. Vaccinees immunized with FI-RSV developed a high titer of serum antibodies to the F glycoprotein of the virus, but these antibodies exhibited a low level of neutralizing activity that was not sufficient to provide effective resistance (200). This was demonstrated later in an experimental setting; passive transfer of antibodies with such low neutralizing activity failed to protect rodents from RSV infection (52). In addition, immunogenicity of the RSV G glycoprotein component of FI-RSV was also adversely affected because young infants developed a low level of antibodies to this other major RSV protective antigen (200).

Third, shortly after the immunization phase of the vaccine trials was completed, it was observed that lymphocytes from the young RSV vaccinees exhibited an exag-

gerated proliferative response to RSV antigens *in vitro* that was significantly greater than that detected after RSV infection (131). Although 27 years ago it was not possible to identify the specific lymphocyte subset that participated in the exaggerated proliferative response to RSV antigens, it is now thought likely that the vaccine induced a high level of virus-specific memory T-lymphocytes, probably of the $CD4^+$ lineage. Recent studies have shown that $CD4^+$ T cells predominate in the pulmonary infiltrate that develops after RSV challenge of FI-RSV–immunized mice (53). On the other hand, studies in mice indicate that FI-RSV vaccine does not induce an RSV-specific $CD8^+$ CTL response (53). These observations suggest that the vaccinees also experienced an imbalance in their cell-mediated immune response in which the $CD4^+$ component predominated, whereas the $CD8^+$ component was deficient (38,53). Finally, an additional imbalance attributable to immunization with FI-RSV was identified recently, namely an alteration in the Th_1 and Th_2 components of the $CD4^+$ response to RSV infection. Ordinarily Th_1 cells predominate in the lungs of RSV-infected mice, but in animals previously immunized with FI-RSV the ratio of the Th_1 to Th_2 $CD4^+$ T cells in the lungs of RSV-infected mice appeared to be reversed and sharply skewed to favor the latter (54,94).

A consideration of the unbalanced immune responses induced by inactivated RSV vaccine suggests a plausible scenario for the pathogenesis of the exaggerated disease that occurred when vaccinees became infected with RSV. Vaccinees were susceptible to infection of their entire respiratory tract because FI-RSV did not induce protective levels of either local respiratory tract or serum-neutralizing antibodies. In contrast, RSV-specific $CD4^+$ memory lymphocytes were present in high concentration at the time infection occurred and were thus available to undergo rapid amplification. In this situation, rapid amplification of RSV $CD4^+$ lymphocytes could occur because virus replication was not adequately restrained by protective levels of serum or local neutralizing antibodies or by the other arm of the cellular immune system, namely $CD8^+$ CTLs, which plays an important role in resolution of infection. As a consequence, an increase in the number of $CD4^+$ T cells infiltrating the sites where RSV was replicating could contribute directly to an enhancement of pulmonary pathology. Imbalance within the $CD4^+$ T-cell compartment might also contribute to this enhancement. For example, in mice, Th_1 $CD4^+$ T cells, which ordinarily predominate in the pulmonary infiltrate that develops during RSV infection, were replaced by Th_2 $CD4^+$ T cells (54,94). The resulting altered pattern of cytokine expression also may have contributed to the observed augmentation of disease associated with administration of FI-RSV.

Several lessons were learned from the potentiation of disease by FI-RSV. First, the chemical treatment and physical purification procedures used for the preparation of each new inactivated vaccine should be evaluated for their effect on the immunogenicity of the major viral protective antigen(s). The ability of a candidate vaccine to induce a high titer of serum antibodies with relevant functional activity, usually neutralization of virus infectivity, which is equivalent to that induced by infection, would constitute a sign that the protective antigen(s) had not been perturbed significantly. Retention of immunogenicity of the major viral protective antigen(s) present in the vaccine should preclude an unbalanced immune response deficient in protective antibodies. Second, evidence for an imbalance involving the $CD4^+$ T-cell response to the vaccine should be sought in experimental animals. An exaggerated $CD4^+$ response, especially in the absence of an adequate protective antibody response, would represent a danger signal that other methods for inactivation of virus infectivity or for purification of virus particles or proteins should be investigated before proceeding further in vaccine development. Third, the potential dangers inherent in pursuing a strategy for selective induction of T-cell responses in the absence of a protective antibody response should be evaluated in experimental animals, if possible. Failure to do so could result in the preparation of an inactivated vaccine that caused potentiation of disease rather than its prevention or amelioration. This caution applies not only to $CD4^+$ Th cells but to $CD8^+$ CTLS because adoptive transfer of the quantity of polyclonal or cloned CTLS required for rapid resolution of infection can cause disease in the recipient and in some circumstances may kill the host (33).

Potentiation of disease by an inactivated virus vaccine is not limited to vaccines containing measles virus or RSV. For example, administration of an inactivated caprine lentivirus vaccine has also been associated with an accelerated, more severe disease in animals subsequently challenged with virus (173). Also, immunization with a subunit vaccine, but not an inactivated whole-virus vaccine, of equine infectious anemia virus (EIAV) enhanced disease caused by subsequent challenge with a heterologous EIAV strain (114). The immunologic mechanisms responsible for these two examples of enhanced disease were not identified.

Another disadvantage of nonliving vaccines is that parenteral immunization against viruses that infect and cause disease that is limited primarily to a mucosal surface may not be completely effective because of the failure of parenterally administered antigen to stimulate a satisfactory local IgA antibody response. Often, parenteral immunization does not stimulate local immunity as effectively as it stimulates systemic immunity (194). This means that resistance in the upper respiratory tract or in the intestines may be less complete than that conferred by local immunization or infection.

Inactivated vaccines also may be at a disadvantage in relation to live attenuated virus vaccines with respect to CTLS response. This type of response is induced most efficiently by processed peptides derived from endogenously synthesized viral proteins that are presented to the immune system in association with MHC class I proteins. There-

fore, it is not surprising that inactivated influenza virus is considerably less effective than virus infection in stimulating a primary CTL response in mice (302). However, inactivated influenza A virus or purified surface glycoproteins derived from it can stimulate a secondary CTL response in sensitized mice or humans (1,74,75,302). As described earlier in this chapter, methods to induce effective durable immunity to CTL epitopes are not currently available.

The Guillain-Barré syndrome was associated with widespread use of inactivated influenza A H1N1 (swine) virus vaccine during 1976 and 1977 (258,259). This indicates that unanticipated, delayed, untoward side effects can be induced by nonliving-virus vaccines. Surprisingly, this syndrome has not been associated with subsequent nonliving influenza virus vaccines, although these products were prepared in the same manner as the 1976 vaccine (259).

It has recently been recognized that antigenic changes can occur during selection and amplification of influenza viruses in the allantoic cavity of embryonated eggs, the substrate used to produce virus for inactivated vaccine. These changes were first recognized by analysis of reactivity patterns of viruses grown in mammalian cell culture (MDCK cells) or eggs with a panel of HA-specific monoclonal antibodies (253). The genetic basis for these mutations involved host cell selection of mutants that replicated more efficiently in eggs. These variants developed a mutation in or near the receptor-binding pocket of the HA that altered their antigenicity and increased their efficiency of replication in eggs (127,128,233,234). The possibility was raised that inactivated vaccine preparations containing such variants might induce an antibody response that was less effective against viruses that were produced in the respiratory tract of humans and whose phenotype would be expected to be similar to that of mammalian cell culture (MDCK) grown viruses (253). Studies in animals and humans suggest that this is not necessarily the case, but the possibility for a substantial decrease in immunogenicity in future vaccine virus strains requires that surveillance be maintained (101,127,238).

Another potentially troublesome situation has been described for inactivated poliovirus vaccine (237). Poliovirus grown in tissue culture contains an uncleaved outer capsid protein VP1 that has limited ability to induce antibodies that neutralize viruses containing a VP1 that has been cleaved by intestinal proteases. This creates a potential problem because the uncleaved VP1 antigenic site (site 1) of poliovirus type 3 appears to be immunodominant over antigenic sites 2 and 3. Thus, the major immune response to inactivated type 3 poliovirus vaccine is directed at the intact antigenic site 1, a site not present on type 3 poliovirus that has been modified by an intestinal trypsinlike enzyme that cleaves this site. Infection by attenuated vaccine or wild-type poliovirus type 3 induces antibodies capable of neutralizing both cleaved and uncleaved virus efficiently. Presumably, during virus replication in the gastrointestinal tract, both cleaved and uncleaved viruses are present and induce an antibody response to both forms of virus. These observations with inactivated influenza and poliovirus vaccine suggest that inactivated vaccine virus derived from tissue cultures or eggs may not mimic the full array of virus forms generated during infection of the host. Thus, immunization with such antigens may induce antibodies that are less effective in neutralizing the total repertoire of viruses produced during infection than are antibodies induced by infection.

Immunization of monkeys with formalin-inactivated SIV produced in human cells induces antibodies directed at antigens present in the cells used to produce the virus (265). These anticellular antibodies can neutralize the infectivity of SIV produced in human cells, but not that produced in monkey cells, by interacting with human antigens incorporated into the virions. This form of neutralization, which is mediated by complement-mediated virolysis, can yield a false overestimate of vaccine immunogenicity. Hence, every effort should be made to avoid immunization with foreign host cell antigens incorporated into virions, which can obscure and confuse interpretation of vaccine immunogenicity (265).

CURRENTLY LICENSED LIVE-VIRUS VACCINES

Except for vaccinia virus, each of the currently licensed live vaccine viruses (Table 1) has a clear lineage. In each instance, the latter strains are of human origin. The adenovirus vaccine strains (types 4 and 7) are wild-type human viruses that produce an asymptomatic infection by virtue of their mode of administration and restriction of replication to a site at which disease does not occur (37). One of the most widely used vaccine strains is a naturally occurring attenuated human poliovirus that was identified by its lack of virulence for the brain and spinal cord of monkeys (type 2 poliovirus, strain 712) (248). Finally, the remaining vaccine viruses were derived from wild-type human viruses by serial passage in cell cultures prepared from an unnatural host, leading to the emergence of mutants that were partially restricted in humans at the portal of entry and/or the target organ(s). In this manner, attenuated mutants of rubella virus and types 1 and 3 poliovirus were selected after passage in monkey kidney tissue culture (80,248). Vaccine strains of yellow fever virus (17D strain) and measles virus were generated in chick embryo cell culture, whereas embryonated eggs were used for attenuation of mumps virus (80).

For the yellow fever virus and poliovirus vaccines, it was possible to identify promising vaccine strains by their attenuation for experimental animals. In monkeys, the 17D strain of yellow fever virus exhibited decreased tropism for the liver, and this served as a sign that this mutant might

be suitably attenuated for humans (287,288). This was confirmed during subsequent clinical trials. In a similar fashion, naturally occurring and tissue culture–passaged strains of poliovirus were evaluated for neurovirulence in monkeys by intraspinal inoculation (245,248). This system was selected because a number of observations suggested that the monkey's spinal cord was more permissive than that of humans to the neuronolytic effect of poliovirus (245,248). Viruses least neurovirulent for monkeys were identified, and these strains were then cloned and recloned by the plaque technique to yield progeny with the lowest possible level of monkey neurovirulence. These candidate vaccine strains were then subjected to additional cycles of selection, which led to the identification of mutants that replicated with high efficiency in the intestinal tract without significant increase in neurovirulence for nonhuman primates. These viruses were then evaluated in clinical trials and shown to be satisfactorily attenuated and immunogenic.

Experimental systems for evaluating virulence were not available for the other vaccine viruses. Hence, these viruses were tested for attenuation directly in humans. Initial preparations of measles and rubella vaccines were not suitably attenuated, but further passage of rubella virus in monkey kidney cell culture and selection of a cold-adapted, temperature-sensitive mutant of measles virus yielded satisfactory vaccine strains. Attenuation of mumps virus passaged in eggs was also monitored in humans. Interestingly, the Jeryl Lynn strain of mumps virus vaccine has recently been found to be a mixture of two closely related strains of mumps virus (4).

Poliovirus and adenovirus vaccines are administered orally, whereas yellow fever, rubella, measles, and mumps virus vaccines are given parenterally. It should be noted that, except for adenovirus vaccine, each of the currently licensed live-virus vaccines is directed against a virus that has a complex pathogenesis of infection in which virus is introduced by inoculation (yellow fever) or by implantation on a mucosal surface (measles, mumps, rubella, and poliomyelitis) and then spreads systemically to the target organ(s).

Basis for Attenuation

Mutants selected by passage in an unnatural host accumulate many mutations, making it difficult to define in a precise manner the genetic basis for their attenuation. In a sense, these satisfactorily attenuated mutants are the product of a process of genetic roulette followed by selection of mutants with the desired properties of attenuation and immunogenicity. The unpredictability of this process is illustrated by the failure of Theiler (287) to produce additional attenuated mutants of yellow fever virus using the protocol that yielded the satisfactorily attenuated 17D strain of virus. The genetic basis for attenuation of measles, mumps, rubella, yellow fever, and vaccinia viruses is unknown, whereas the genetic determinants of attenuation of the three live-poliovirus vaccine strains have been characterized extensively.

The poliovirus vaccine strains are temperature sensitive with respect to replication and exhibit greatly decreased neurovirulence in monkeys after intraspinal administration of virus (245). Because the poliovirus vaccine viruses replicate efficiently in the gut of vaccinees and can produce viremia, it is likely that restriction of replication in neurons represents the basis of attenuation of these viruses for the CNS of humans, monkeys, and rodents. The genetic basis of this neuroattenuation of each of the three poliovirus vaccine strains has been partially defined (161,278,282). The identification of mutations contributing to neuroattenuation of the three vaccine viruses derives from a large series of studies involving (a) sequence analysis of virulent parental type 1 and type 3 viruses and their attenuated vaccine strain derivatives; (b) sequence analysis of neurovirulent "revertant" virus isolated from vaccine-associated cases of poliomyelitis (for types 1, 2, and 3); (c) *in vitro* generation of chimeric recombinant viruses produced from cDNA clones of virulent and attenuated viruses or viruses bearing site-specific mutations thought to be associated with neuroattenuation on the basis of sequence analysis of revertant viruses; and (d) evaluation of the neurovirulence of these naturally occurring or *in vitro*–generated viruses in monkeys, normal mice, or mice transgenic for the human poliovirus receptor. These analyses have identified an attenuating nucleotide substitution mutation in the same site in the 5' noncoding region of each of the three vaccine viruses (nucleotide 480 in type 1 virus, 481 in type 2 virus, and 472 in type 3 virus), a missense mutation in the VP1 capsid protein of poliovirus type 2 and the VP1 and VP3 capsid proteins of type 3, and a mutation in the 3D region polymerase of type 1 vaccine virus. It is likely that other mutations in the Sabin vaccine viruses, especially the Sabin type 1 virus, also contribute to attenuation for humans. The mutation in the 5' noncoding region (a) alters the predicted secondary structure of this important regulatory region, (b) is responsible in part for the *ts* phenotype of the vaccine viruses, and (c) decreases virus replication *in vitro* in cells of neural origin (160). An assembly defect in the capsid results from an attenuating mutation in VP1 of PV2 (159). These mutations can now be monitored during manufacture (42) and during replication *in vivo* (6). Importantly, reversion in several of these attenuation sites has been detected during the *in vitro* passage of virus that is required for the production of vaccines (42) or after replication *in vivo*, even as early as 47 hr after vaccine administration. Specifically, the rare reversion of the attenuating mutation at nucleotide 472 of the 5' noncoding region of the Sabin type 3 vaccine strain that occurs during vaccine production can now be detected using

sensitive polymerase chain reaction techniques. Vaccine lots with an increased frequency of reversion at residue 472 exhibit increased neurovirulence, and such lots can be discarded as unacceptable for human use (42). Thus, it is now possible to use molecular virologic techniques to control the manufacture of vaccines by identifying the presence of the desired attenuating mutations as well as the absence, or low frequency, of unwanted reversions (42).

The attenuated yellow fever virus vaccine strain 17D, which was derived from its virulent Asibi virus parent after 234 passages in chick embryo cell culture, has been sequenced, and the deduced amino acid sequence has been compared with that of the parental strain (96,185,287). The Asibi and 17D polyproteins differ at 32 amino acid positions, which are not uniformly distributed throughout the genome (96). The envelope glycoprotein contains 12 of the 32 changes (40%), although it represents only 12% of the total viral protein sequence. Many of the amino acid substitutions in the envelope glycoprotein are not conservative. Additional studies are needed to identify the specific changes associated with attenuation. However, it should be noted that for each of the attenuated vaccine strains analyzed (poliovirus types 2 and 3 and yellow fever virus), suggestive or definitive evidence has been obtained for a role of altered viral surface proteins in attenuation (254). This is consistent with other observations that an amino acid substitution in a surface protein of an icosahedral or enveloped virus can have profound effects on virulence and tissue tropism (56,66,266,267).

A different approach was taken to develop live-adenovirus vaccines that are currently licensed for use in military personnel, where adenoviruses cause epidemic respiratory tract disease (37,72). Attenuation is achieved by limiting infection to an area of the body in which disease does not develop. The vaccine contains wild-type virus that is administered orally in an enteric coated tablet. Virus is released when the tablet enters the intestines. There it establishes an asymptomatic, immunizing infection that does not spread to the respiratory tract, the region in which disease develops during natural infection. Although vaccine virus is shed in feces, spread to susceptible contacts occurs with a very low frequency. Selective enteric infection stimulates a vigorous systemic immune response that confers complete or almost complete resistance to adenovirus disease (72). Resistance to reinfection is also impressive (70%) but not complete (72,262).

Advantages of Live-Virus Vaccines

The major advantage of live-virus vaccines is the activation of all phases of the immune system yielding a balanced response, systemic and local, Ig- and cell-mediated. This is particularly important for infections in which cell-mediated immunity plays an important role and for mucosal infections in which both local and systemic immunity are required for optimal resistance. Topical infection with a live-virus vaccine is usually more effective in stimulating a local response in the unprimed host than is a nonliving-virus vaccine administered parenterally (24,166,194, 213–216). Live-virus vaccines also stimulate an immune response to each of the protective antigens of a virus, and this obviates the difficulties that arise from selective destruction of one of the protective antigens that may occur during preparation of a nonliving-virus vaccine and that can result in disease potentiation. Furthermore, immunity induced by live-virus vaccines is generally more durable and more effective (120). It should be noted that there are also practical advantages to many live-virus vaccines, such as low cost of production and ease of administration.

Disadvantages of Live-Virus Vaccines

The potential for contamination with live adventitious agents always exists. Fortunately, this has rarely been a problem. Some of the early lots of live-poliovirus vaccine were contaminated by live SV40, but this virus was quickly removed from the vaccine (109). The live yellow fever virus vaccine was initially contaminated with an avian leukosis virus; this contaminant was recently removed from virus seed preparations, and all subsequent vaccine lots have been free of this adventitious agent (254). Follow-up of individuals who were given live vaccine contaminated with SV40 or avian leukosis virus has failed to identify any long-term adverse effect, including cancer, associated with exposure to these adventitious viruses (191,301). Nonetheless, prudence dictates that live-virus vaccines should be free of such contaminants.

Some live-virus vaccines, such as the measles virus, rubella virus, and yellow fever virus vaccines, retain a low level of residual virulence. The reactions produced by these vaccines are minor and have not interfered with acceptance and widespread use of these products. A more serious problem is that of restoration of a varying degree of virulence during infection by vaccine virus. This occurs with the poliovirus vaccine at an extremely low frequency, i.e., about one in 10^6 to 10^7 immunizations (208). Most vaccine-associated cases of paralysis in vaccinees or their close contacts occur after the first dose and involve the type 3 vaccine strain (208). A significant proportion of the paralytic illnesses associated with poliovirus vaccine occurs in individuals who are immunocompromised, but this may not represent a manifestation of genetic alteration of vaccine virus (208). In most instances, however, vaccine virus appears to have regained neurovirulence by mutation, allowing it to produce disease in the vaccinee or a close contact (188). In addition to mutation, polioviruses with recombinant genomes have been isolated from vaccinees with paralytic disease, suggesting that restoration of virulence might occur by the process of recombination as well as by mutation (89).

The genetic basis for the very rare increase in virulence of the poliovirus vaccine strains during replication *in vivo* is relatively well understood. However, a paradox exists in that mutation toward a higher level of virulence for the CNS occurs rapidly and frequently during replication of type 3 poliovirus in the intestinal tract of vaccinees, but vaccine-associated paralytic disease is extremely rare (6, 183,187,189). It is now clear that mutations that increase neurovirulence of the virus for primates can occur in each of the three Sabin vaccine strains during infection of vaccinees (161,278,282), but fortunately, poliomyelitis in vaccinees or contacts is rare (189). Surprisingly, the mutations that are associated with restoration of virulence can be detected during the manufacture of the vaccine, and viruses bearing these mutations are further selected during virus replication *in vivo* (42,282). Genetic instability was also a problem with the 17D yellow fever virus vaccine during early field trials. Encephalitis was observed in 1% to 2% of young vaccinees administered virus that had been passaged 20 times beyond the original seed lot (86,288).

Another potential difficulty with live-virus vaccines is persistent infection by vaccine virus. This occurs in immunodeficient children given live-poliovirus vaccine and may lead to paralytic disease (310). It had been reported that rubella virus can be recovered from the lymphocytes of immunologically normal individuals with arthritis up to 6 years after immunization or natural infection (39,40). The frequency and importance of this phenomenon remain to be defined. Of interest, persistence of measles vaccine virus has not been detected, nor has the vaccine virus been implicated in subacute sclerosing panencephalitis (180). Instead, widespread use of live measles vaccine has almost eradicated this rare but serious sequela of persistent infection with wild-type measles virus. Although there is a theoretical risk that infection of the fetus with rubella vaccine virus during the first trimester of pregnancy might lead to development of the congenital rubella syndrome, the actual risk appears to be negligible (190).

In certain circumstances, naturally occurring wild-type viruses may interfere with infection by a live-virus vaccine resulting in a decrease in vaccine efficacy. This has been observed primarily with live-poliovirus vaccine strains that are subject to interference by a wide variety of intercurrent enteric viruses. This is one of the reasons why multiple doses of polyvalent live-virus vaccines are required to assure a protective immune response to each component.

Defective interfering particles have been identified in one preparation of live measles virus vaccine (32). The presence of such particles could modify the clinical response to vaccine and possibly produce variation in immunogenicity and level of attenuation among successive lots of vaccine.

Finally, it should be noted that stability is a serious problem with labile vaccine viruses such as measles virus. The need for storage and transport of measles vaccine at low temperature (4°C) has limited its usefulness in some tropical areas where maintenance of a "cold chain" for transport and storage is difficult.

DIRECTIONS FOR FUTURE DEVELOPMENT OF LIVE ATTENUATED VIRUS VACCINES

Conventional Approaches and Naturally Occurring Attenuated Mutants

Conventional techniques for attenuation of viruses such as passage of virus at low temperature, mutagenesis followed by selection of mutants with the desired phenotype(s), or passage of virus in heterologous tissues will continue to have a role in the development of live-virus vaccines. These techniques have been used to generate candidate vaccine strains for varicella virus, cytomegalovirus, ortho- and paramyxoviruses, alphaviruses, flaviviruses, HAV, and arenaviruses. In the case of RSV, a candidate vaccine strain that was partially attenuated for susceptible young infants by multiple *in vitro* passages at low temperature in embryonic bovine kidney cells was subsequently subjected to chemical mutagenesis, and fully attenuated derivatives containing attenuating *ts* or small plaque mutations were identified (197). In this way, two conventional approaches were combined to introduce different classes of attenuating mutations, e.g., host range mutations introduced by passage in a heterologous host and *ts* mutations introduced by chemical mutagenesis.

Variation in virulence of naturally circulating human viruses has been documented, but this phenomenon has not been studied systematically nor has its implications for immunoprophylaxis been adequately explored. Almost 40 years ago, Sabin (244,245) observed that strains of poliovirus recovered from healthy individuals exhibited a wide range of neurovirulence as measured by intracerebral or intraspinal inoculation of monkeys. Approximately one fifth of these strains were highly attenuated. This suggests that systematic study of viruses recovered from healthy persons might identify naturally occurring attenuated mutants of viruses other than poliovirus. It should be noted that the type 2 poliovirus strain of the live oral vaccine was identified in this manner.

The use of conventional methods for achieving attenuation will diminish as the newer techniques for construction of viruses with defined mutations or gene constellations are applied to development of live-virus vaccines. Viruses bearing stable, defined, identifiable attenuating mutations or gene constellations represent the vaccine strains of the future because the genetic basis for attenuation will be known and can be monitored directly during all phases of vaccine development, manufacture, and utilization in humans.

Attenuating Mutations Introduced into Coding Regions

Missense

A missense mutation that specifies an amino acid substitution in the encoded viral protein can decrease its function *in vivo*, thereby attenuating the virus for its host. Three classes of missense mutants with easily identifiable phenotypes have been evaluated extensively for their level of attenuation and their potential usefulness in live attenuated viral vaccines: (a) *ts* mutants, (b) protease activation (*pa*) mutants, and (c) mutants with altered interaction with host cell receptors.

ts mutants are conditional lethal viruses that replicate efficiently at permissive temperatures (32–34°C) but are restricted in replication at the high end of the normal temperature range (37–39°C). *ts* mutants can be grown efficiently *in vitro* at the permissive temperature for vaccine production because the attenuating *ts* mutations restrict replication only at elevated temperature. *ts* mutations that attenuate have been identified in most, if not all, genes of the viruses that have been examined systematically (261), and multiple sites within a given protein can sustain ts mutation (148). Thus, it is possible to introduce a *ts* mutation into almost any gene of a vaccine virus or, alternatively, several mutations can be introduced into the same gene. *ts* mutants of respiratory viruses (eg., influenza A virus and RSV) were sought with the expectation that their *ts* defect would restrict virus replication in the warmer lower respiratory tract (37°C), the major site of disease; however, sufficient replication should occur in the cooler upper respiratory tract to immunize the host. This was indeed the case because *ts* mutants of respiratory viruses were observed to be more restricted in replication in the lungs than in the cooler upper respiratory tract. Importantly, virus replication in the latter site was sufficient to immunize the host against infection with virulent virus. However, genetic instability has presented a problem for *ts* mutants because there is strong selection *in vivo* for ts^+ viruses that have undergone reversion to wild-type sequence or sustained suppressor mutations (290). Fortunately, it is now possible to introduce site-specific *ts* mutations into the influenza A virus genome, and it is likely that a sufficient number of *ts* mutations can be introduced to yield a genetically stable *ts* vaccine virus (272).

The second approach to producing missense mutations that specify attenuation involves the isolation of *pa* mutants. Proteolytic cleavage of the F glycoprotein of paramyxoviruses is required for activation of infectivity, and this process is thought to play an important role in tissue tropism and pathogenicity. Trypsinlike enzymes that are present in host tissues are required for the cleavage of the fusion protein precursor F0 to its disulfide-linked F1 and F2 subunits. *pa* mutants of murine parainfluenza virus type 1 (Sendai virus) were selected by growing virus in the presence of another protease such as chymotrypsin or elastase (111,280,281). *pa* mutants selected in this manner were resistant to activation by trypsin but formed plaques in the presence of the selecting protease. These *pa* mutants sustained amino acid substitutions that abolished the cleavage site for trypsin and created a new site for cleavage by chymotrypsin or elastase (111,115). In this manner a new N-terminus of F1 was generated. The *pa* mutants were shown to be attenuated in mice as indicated by an increase in their LD_{50} (lethal dose) and a decrease in pulmonary histopathology. Also, the *pa* mutants were immunogenic and induced resistance to wild-type virus challenge; however, they were not completely stable during replication *in vivo* (111). The trypsin-sensitive revertant virus that emerged during infection *in vivo* exhibited a restoration of pneumovirulence for mice (184).

Viral surface proteins can also undergo mutation that results in attenuation. This is not surprising because missense mutations affecting surface proteins can alter tissue tropism and pathogenesis of infection (266). This was shown most convincingly in studies in which mutants were selected by growing virus in the presence of a monoclonal antibody that neutralized virus infectivity and by isolating antigenic mutants that were no longer neutralized by the selecting antibody. These variants are referred to as monoclonal antibody–resistant (*mar*) mutants. Generally, such mutants sustain only a single amino acid substitution. *mar* variants of reovirus with a mutation in the S1 capsid protein, which is responsible for adsorption to host cells, exhibit decreased tropism for specific regions of mouse brain but grow normally in visceral organs (266,267). Similar observations have been made for other viruses. It is thought that the mutation that abrogates neutralization by antibody can also affect the interaction of the surface protein of the *mar* mutant with its specific host cell receptor. Attenuation results if the virus is no longer able to enter and infect cells in the target organ in which the virus produces disease. The usefulness of *mar* mutants as candidate vaccine viruses for humans remains to be demonstrated because reversion to virulence after replication of *mar* mutants *in vivo* may occur with high frequency (83). However, it has been possible to introduce multiple mutations sequentially into the capsid proteins of coxsackievirus B4 and to effect a stepwise reduction in virulence (226). In addition, it has been possible to introduce mutations into the surface E2 protein of an alphavirus that attenuate the virus (62,257). Importantly, it was shown that a specific locus and a specific codon can be selected for site-directed mutagenesis that will maximize attenuation and minimize restoration of virulence by reversion or suppression (257).

Because only a small number of mutations appear to be sufficient for attenuation in certain circumstances (e.g., three mutations in the Sabin poliovirus type 3 vaccine strain), it should now be possible using modern molecular techniques to introduce the requisite number of attenuating missense mutations into a virulent or partially attenu-

ated virus in order to produce a vaccine virus that has a stable attenuation phenotype.

Deletions

Deletion mutations that represent either partial or complete deletion of a given protein sequence can attenuate a virus for its host. Deletion mutations can be used in several ways to construct candidate vaccine viruses. First, deletion mutations can be produced in one or more genes to attenuate the virulence of the virus for its host. Second, a protein that mediates a specific function in the host that would be undesirable in a vaccine virus can be deleted. The use of deletion mutations as the basis for the attenuation of a vaccine virus has theoretical appeal because this form of mutation cannot be corrected directly because this would require restoration of deleted sequence. Although phenotypes specified by deletion mutants can be modified by suppressor mutation (18,291), attenuation specified by an appreciable deletion is more stable than that specified by a single missense mutation.

To be useful in construction of live vaccine viruses, deletion mutations should restrict virus replication *in vivo* while permitting virus to grow efficiently *in vitro* during vaccine production. This is most easily achieved for poxviruses, herpesviruses, and adenoviruses, whose large genomes encode a number of functions that are not essential for virus replication *in vitro*. Nonetheless, these functions favor virus replication *in vivo* through evasion of host defense mechanisms or by other strategies that remain to be defined (16). The NYVAC mutant of vaccinia virus is an example of a candidate vaccine strain that has been attenuated for humans by deletion of 18 open reading frames that are not required for efficient growth of virus in cell culture (279).

Attenuating deletion mutations can be designed to abrogate specific undesirable functions of a virus. For example, HSV can cause persistent infection in humans by initiating a latent infection in dorsal root ganglion neurons. After reactivation of the virus present in the latently infected neurons, HSV travels down the nerve axon and replicates in the skin enervated by the neuron. Thus, one goal of HSV vaccine development is elimination of the function that is essential for virus replication in dorsal root ganglion neurons so that vaccine virus is not able to establish latency. Recently, this was achieved by deletion of a 34.5-Kd protein that is required for efficient replication of HSV in neurons (306). Thus, directed deletion of a gene involved in tropism for the CNS greatly diminished the ability of the mutant to establish latency and to be reactivated from latency, i.e., properties desirable for a live attenuated HSV vaccine. A second example of a directed mutation is the deletion of the intergrase protein of SIV, rendering the virus incapable of establishing persistent infection (298). In this case, viral antigens are expressed from episomal DNA during a self-limited infection.

It has been possible to introduce attenuating deletion mutations into a number of genes of positive sense RNA viruses (60), but it has only recently been possible to accomplish this for a segmented, negative-sense RNA virus, namely influenza A virus (91). This was accomplished by creating the desired mutations in a cDNA copy of an RNA virus gene and then transcribing RNA copies of the mutant DNA. These RNA transcripts were then transfected into cells that yielded infectious virus bearing the gene with the introduced mutations (34). Over the next decade it is likely that the creation of deletion mutations will be widely used as a strategy to produce stable live attenuated virus vaccines.

Insertions

A virulent virus can be attenuated by the insertion of genes with known antiviral activities or with known immunoregulatory functions. Such recombinant viruses encode foreign proteins that function to restrict replication of the virus, and this results in attenuation of the virus for its host. Viruses bearing such attenuating foreign immunomodulatory proteins have been found to be satisfactorily attenuated even for immunodeficient animals. Interferon γ, IL-2, the interferon-induced double-stranded RNA-activated p68 protein kinase, tumor necrosis factor, and IL-10 have each been shown to attenuate vaccinia virus for mice (103,138,143,149,250). This mechanism of attenuation is only suitable for the large DNA viruses that can accommodate a large amount of foreign genetic material. The approach is particularly promising as a method to rapidly attenuate a virus and derive a vaccine suitable for use in immunodeficient individuals.

Attenuating Mutations Introduced Into Noncoding Regions

It is possible to attenuate a virus for its host by introduction of mutations into the 5' or 3' noncoding regions of the genome. These noncoding regulatory regions contain *cis*-acting signals required for efficient replication, transcription, and translation that represent target sites for modification of these functions by mutation. As mentioned earlier, each of the poliovirus vaccine strains has an attenuating nucleotide substitution mutation at the corresponding site in its 5' noncoding region. These mutations have a tissue-specific effect that decreases virus replication in neurons *in vivo* while at the same time permitting efficient virus replication in cell culture used for vaccine production. It has been suggested that host cell proteins interact with the 5' noncoding region of the poliovirus genome and that this essential interaction occurs efficiently for vaccine mutants in cell culture or intestinal tissue *in vivo* (where vaccine virus grows to high titer) but is highly restricted in CNS neuronal cells. This tissue-specific effect in which the virus is restricted in replication in the target organ is a highly desirable property for this vaccine virus because it permits

efficient production of virus in cell culture as well as the high level of replication in the intestines that is required for induction of effective immunity.

Attenuating deletion mutations in 5' or 3' noncoding regions of an alphavirus also have been produced (139). Several of these attenuated mutants replicate reasonably well in mouse cells but are highly restricted in their growth in mosquito cells. These observations suggest the possibility that a live-virus vaccine containing such host-range mutations could interrupt the mosquito–veterbrate–mosquito transmission cycle. The presence of attenuating deletion mutations in both the 5' and 3' noncoding regions of the alphavirus specified a marked degree of attenuation for the CNS of mice.

Another strategy for producing attenuating mutations in the 5' and 3' noncoding regions involves substitution of one or both of these regions from a partially related but heterologous virus. The 5' and 3' noncoding ends of an influenza B virus gene were used successfully to replace the corresponding ends of an influenza A virus NA gene (201). In this situation, the influenza A virus polymerase proteins had to recognize and react with the moderately divergent 5' and 3' ends of the influenza B virus gene. As a consequence of this inefficient interaction, the influenza A virus bearing such a chimeric NA gene was highly attenuated for mice. Similar exchange mutants between two alphaviruses also generated attenuated chimeric viruses (140).

Host-Range Mutations: The Jennerian Approach

The Jennerian approach to the development of live attenuated viruses involves the use of a virus strain of mammalian or avian origin to immunize humans against a human virus that is related antigenically to the animal or avian strain. Mammalian and avian viruses that are well adapted to their natural host often do not replicate efficiently in humans and hence are attenuated. At present, we lack a thorough understanding of the genetic basis for this form of host-range restriction. However, those mammalian or avian viral genes that have been identified as being responsible for host-range restriction in humans exhibit significant divergence of nucleotide sequence from that of the corresponding human viral genes. This means that ultimately it should be possible to delineate host-range restriction at the molecular level. In order to attain this level of understanding, current efforts to develop vaccines using the classical approach of Jenner have been updated to incorporate the techniques of contemporary viral genetics, molecular biology, and immunology. Human parainfluenza virus type 3 (PIV-3) is a paramyxovirus that is second in importance only to RSV as an etiologic agent of serious pediatric viral respiratory tract disease. The two surface glycoproteins of PIV-3, designated HN and F, each serve as a protective antigen. Bovine PIV-3 is antigenically related to human PIV-3. For example, the prototype strains of bovine and human PIV-3 differ only four-fold in reciprocal hemagglutination inhibition and neutralization tests (48). The level of homology of the human and bovine PIV-3 sequences of the F, HN, NP, P, C, and M proteins varies from 62% to 86% (249,274). Infection of squirrel monkeys with bovine PIV-3 induces a moderate titer of neutralizing antibodies to human PIV-3, and these animals exhibit significant resistance in their lower respiratory tract to subsequent challenge with human PIV-3 (48). Also, the bovine PIV-3 was attenuated for the lower respiratory tract of rhesus monkeys and chimpanzees. Although the bovine PIV-3 is restricted in replication in rhesus monkeys and chimpanzees, it grows well enough to induce a serum-neutralizing antibody response that should provide resistance to human PIV-3 infection. These encouraging observations provide a rationale for clinical evaluation of bovine PIV-3 as a live vaccine for prevention of human PIV-3 disease.

Another example of the Jennerian approach is a mammalian rotavirus strain, rhesus rotavirus (RRV), which is under intensive study as a vaccine candidate (124). This simian rotavirus has not been recovered under natural conditions from humans and is thus not a virus of human heritage. Although the genes of RRV exhibit significant divergence in sequence from the corresponding genes of human rotaviruses, this simian rotavirus is similar to human rotavirus type 3 when tested by neutralization. RRV has undergone extensive clinical evaluation for safety and efficacy in various studies in the United States and abroad and appears to be satisfactorily immunogenic and able to induce significant resistance to disease caused by human rotaviruses of the same serotype, i.e., serotype 3. Other studies demonstrated that serotype-specific immunity is required for satisfactory protection against rotavirus diarrhea. Thus, there was a need for a multivalent vaccine that would induce resistance to each of the four major human rotavirus serotypes. To meet this need, the Jennerian approach was modified by constructing reassortant viruses, each of which contained 10 RRV genes plus a single human rotavirus gene that coded for the major neutralization antigen (VP7) of serotype 1, 2, or 4. The intent was to prepare single gene substitution RRV reassortants with the attenuation characteristics of this simian virus and the neutralization specificity of human rotavirus serotype 1, 2, or 4. Bovine rotaviruses are also being evaluated for use in a rotavirus vaccine virus (43).

Attenuation by Gene Incompatibility

Viral proteins of the PB2, PB1, and PA polymerase complex of influenza virus function as a unit, performing a variety of biochemical activities. These proteins must be compatible with each other in order to function efficiently. Disruption of this compatibility can bring about attenuation. An example of the use of this strategy is provided by the properties of an influenza A reassortant virus derived from mating an avian influenza A/Pintail/79 virus and a human influenza A/Washington/80 virus. This reassortant,

which possesses a mixed polymerase complex with PB1 and PA genes derived from the human influenza A virus parent and the PB2 gene derived from the avian influenza A virus parent, is attenuated in monkeys (263). Reassortant viruses with this polymerase genotype replicate efficiently in avian tissue but grow poorly in mammalian cell culture. These avian/human influenza A reassortants exhibit an alteration in host range in ovo and *in vivo*; the virus replicates to high titer in the allantoic cavity of chicken embryos, but it is restricted in its growth in the respiratory tract of monkeys. Incompatibility of polymerase genes also appears to contribute to the attenuation of influenza A/PR/8/34 virus reassortants for humans (85). The phenomenon of gene incompatibility resulting in attenuation is also seen with DNA viruses. Intertypic recombinants between HSV-1 and HSV-2 often exhibit decreased neurovirulence for experimental animals compared with either parent (97,118,289).

Antigenic Chimeric Viruses

Recombinant DNA technology has made it possible to construct attenuated chimeric viruses bearing the protective antigens of a virus plus the remaining coding and noncoding regions of another related virus that bears attenuating mutations. A viable chimeric poliovirus bearing the capsid proteins (i.e., the protective antigens) of type 3 virus and the remaining nucleotide sequences of the type 1 vaccine virus has been prepared. This chimera might prove useful in immunoprophylaxis because it combines type 3 serologic specificity with the attenuation and greater genetic stability of the type 1 vaccine virus (135). The properties of this poliovirus chimera demonstrate the feasibility of combining the attenuating mutations present in the noncoding or the nonstructural protein regions of one virus with the protective antigens of a closely related virus. This concept of substituting the protective antigens of one virus onto a genetic background of another related virus containing attenuating mutations has been used successfully in the development of candidate live-virus vaccines for the segmented RNA influenza viruses and rotaviruses (see Chapters 41 and 55).

Live-Virus Vaccines as Vectors

Replication Competent

Stable attenuated vaccine viruses have been used to construct viable recombinants that express the protective antigens of other viruses. To date, the most success has been achieved using vaccinia virus as the recombinant vector (192). This virus has a large genome into which a variety of foreign viral genes can be inserted and expressed without seriously compromising the capacity of vaccinia virus to replicate. Equally important, vaccinia virus has the longest record of successful use as an effective, attenuated live-virus vaccine. Recombinant vaccinia viruses expressing the protective antigen (or antigens) of a large number of viruses have been constructed and shown to be protective in experimental animals. Polyvalent live vaccinia recombinants have been used to successfully immunize experimental animals, even in the presence of immunity to one of the foreign viral antigens (84). This approach to immunization has the advantage that foreign viral antigens are expressed at the cell surface in the context of host histocompatibility antigens, thereby assuring an effective cellular as well as humoral immune response. Vaccinia virus derivatives with greatly reduced systemic pathogenicity for experimental animals or humans have been constructed or identified (273,279). Such highly attenuated derivatives of vaccinia are needed for use in immunoprophylaxis in this era of widespread HIV infection because the vaccinia virus strain used in the smallpox vaccine can cause progressive vaccinia in an immunocompromised host.

Adenoviruses also have the potential to serve as recombinant vectors for construction of live vaccines because they have a large genome with at least one nonessential region as well as several sites into which foreign genes can be inserted and expressed without loss of infectivity (205). In addition, safe, effective live-adenovirus vaccine strains, which produce a silent, selective intestinal infection, are available for use as viral vectors (110). The immunogenicity of adenovirus or vaccinia recombinants bearing viral protective antigens appears to be satisfactory in rodents or dogs, but the responses of chimpanzees and humans have been less promising (59,110,276).

Replication-Defective vectors

Virus vectors capable of replicating to high titer *in vitro* but unable to grow efficiently *in vivo* are being sought for use in inducing humoral and CTL immunity. Such nonreplicating vectors would be safe in the immunosuppressed host. Such vectors undergo an abortive infection *in vivo* but express the foreign protein during this abortive infection and can thereby present antigens efficiently to both the humoral and cellular arms of the immune response. Two vectors have emerged that appear to meet these requirements. First, avian poxviruses, e.g., fowlpox or canarypox viruses, replicate to high titer in avian cells *in vitro* but cause abortive infection in mammals *in vivo* (283). These viruses are able to express early and late viral proteins in abortively infected mammalian cells (264) and can induce humoral and Tc-cell responses *in vivo* (58). Inoculation of dogs or cats with avipox-rabies glycoprotein G recombinant viruses induces resistance to wild type rabies virus challenge (284). The NYVAC (279) and MVA (273) deletion mutants of vaccinia virus also appear to be immunogenic despite the fact that these mutants cause an abortive infection *in vivo*.

The second vector virus being evaluated is a defective adenovirus that lacks the EIA gene. However, the mutant can replicate to high titer in the 293 cell line that constitutively expresses the EIA-encoded proteins. This type of defective adenovirus recombinant is prepared by inserting a foreign gene encoding viral protective antigens into the EIA region and then growing the adenovirus recombinant in 293 cells. Immunization of experimental animals with such replication-defective recombinant adenoviruses has induced resistance in experimental animals to a flavivirus or to Epstein-Barr virus (117,228).

Replication-defective HSVs have been produced in a manner analogous to that of the EIA-deficient adenoviruses (207). However, the replication-defective herpesvirus is being used not as a vector of foreign viral proteins, but as a vaccine to protect against homologous virus infection. An immune response is directed not only to the antigens present in the input virus but also toward viral gene products expressed during the abortive infection *in vivo*. Such a replication-defective herpesvirus could also be used as a vector of foreign viral proteins.

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